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Clinical and Experimental

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CLINICAL AND EXPERIMENTAL

THE DETERMINATION OF EXCHANGE RATES IN THREE-COMPARTMENT STEADY-STATE CLOSED SYSTEMS THROUGH THE USE OF TRACERS

J. S. ROBERTSON, M.D., PH.D., D. C. TOSTESON, M.D.,* AND J. L. GAMBLE, JR., M.D.**

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THE study of exchange rates by the use of tracers requires some mathematical treatment of the data because the relationships between the behavior of the tracer (which can be observed) and that of the carrier substance under study (which must be calculated) lose their simplicity when feedback processes are significant. The purpose of this article is to make available in a form useful to a biologist having a minimal mathematical background the relationships involved in three-compartment closed systems. The three-compartment systems are sufficiently complicated to illustrate the mathematical principles involved, yet simple enough for them to be completely analyzed with experimental access to only one of the compartments being assumed, and with the use of a single species of tracer.

Literature.—The general mathematical basis for the interpretation of tracer experiments in closed steady-state systems has been presented by Sheppard[1] and by Sheppard and Householder.[2] Two cases may be distinguished for the three-compartment system with restricted exchange, depending upon whether

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the investigator has access to an end compartment [1] or [3] or to the middle compartment [2], in the following schematic representation:

$$1 \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} 2 \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} 3$$

The two cases will be referred to as series and as parallel cases, respectively. Gellhorn, Merrell, and Rankin[3] use an analysis in which it is assumed that the parallel case is involved; this is supplemented by Cohn and Brues'[4] treatment of the series case. The system involving direct exchange between compartments [1] and [3],[5] will not be treated here. A more general survey of the literature on multicompartment systems is being published (Robertson, J. S.: The Theory and Use of Tracers in Determining Transfer Rates in Biological Systems, Physiol. Rev., April, 1957. In press).

Definitions and Assumptions.—A tracer may be an agent foreign to the system under study, but in the present considerations it will be defined as a labeled form of a substance normally present. The unlabeled form of the substance will be referred to as the carrier. A fundamental assumption in tracer theory is that the rate constants of the tracer are precisely those of its carrier; exceptions to this assumption do occur, but will not be considered in the present treatment. A compartment is defined as a region or volume in which a uniform concentration of the carrier and uniform mixing of the tracer with the carrier is assumed at all times. The restriction to a consideration of the steady state implies that the volumes of the compartments and the concentrations and exchange rates of the carrier remain constant. It is convenient to use the terminology of radioactive tracers, but the principles apply to all tracers. Solomon[6] discusses the basic assumptions and their implications more thoroughly.

Symbols.—The systems to be analyzed are schematically represented above. The arrows indicate that exchange occurs between compartments [1] and [2] and between compartments [2] and [3], but not directly between [1] and [3]. The symbols to be used in the present analysis are defined as follows:

Symbols	*Definitions*	*Units*
S_n	Amount of exchangeable carrier in n^{th} compartment	Mass
R_n	Amount of tracer in n^{th} compartment	Disintegration rate/mass
x_n	Specific activity of tracer in S_n	Disintegration rate/mass
x_{n0}	Specific activity of tracer in S_n at time zero	Disintegration rate/mass
E	Equilibrium (infinite time) specific activity	Disintegration rate/mass
ρ_{nr}	Rate of exchange of carrier in either direction between n^{th} and r^{th} compartments	Mass/time

k_{nr}	Fraction of S_n transferred to r^{th} compartment in unit time; $k_{nr} = \frac{\rho_{nr}}{S_n}$	$Time^{-1}$
${}_nC_{ij}$	Coefficient of j^{th} exponential term in equation for behavior of the tracer in i^{th} compartment when tracer is initially in n^{th} compartment	Specific activity (may also be mass or concentration)
λ_j	Exponential constant in j^{th} exponential term	$Time^{-1}$

Equations.—With the left-hand member in terms of amounts, the differential equation for the behavior of the tracer in compartment [1] is:

$$\frac{dR_1}{dt} = -\rho_{12}x_1 + \rho_{12}x_2 \qquad 1.$$

Division of the members of equation 1 by S_1 yields equation 2, which is in terms of specific activities. Analogous procedures yield the equations for compartments [2] and [3].

$$\frac{dx_1}{dt} = -k_{12}x_1 + k_{12}x_2 \qquad 2.$$

$$\frac{dx_2}{dt} = k_{21}x_1 - (k_{21} + k_{23})x_2 + k_{23}x_3 \qquad 3.$$

$$\frac{dx_3}{dt} = k_{32}x_2 - k_{32}x_3 \qquad 4.$$

The general solutions of the system of equations 2, 3, and 4 have the form:

$$x_1 = E + C_{11}e^{-\lambda_1 t} + C_{12}e^{-\lambda_2 t} \qquad 5.$$

$$x_2 = E + C_{21}e^{-\lambda_1 t} + C_{22}e^{-\lambda_2 t} \qquad 6.$$

$$x_3 = E + C_{31}e^{-\lambda_1 t} + C_{32}e^{-\lambda_2 t} \qquad 7.$$

When a three-compartment system is involved, plots of the experimental data will yield curves which can be described by equations 5, 6, and 7. The constants (the C's and λ's) of these equations characterize the observable behavior of the tracer in the particular system involved. The constants obtained by experiment in any one compartment may be used to calculate the constants describing the behavior of the tracer in the other compartments and to calculate the constants which describe the kinetics of the carrier, as shown below.

Calculation of Carrier Kinetics From Tracer Behavior.—The formulas used for calculation of the constants which describe the kinetics of the carrier (the S's and ρ's) are different for the parallel and series cases. The formulas given below assume that the investigator has access to one compartment only; simpler formulas obtain if access to two or three compartments is assumed. Derivations of the formulas (in other notations) are available elsewhere.*

*A derivation of the solutions and the formulas, giving the major intermediate steps, will be sent by the senior author, Dr. J. S. Robertson, on request.

Series Case.—In the series case, access to compartment [1] only is assumed, so equation 5 (with numerical values for x_{10}, E, C_{11}, C_{12}, λ_1, and λ_2) is obtained by experiment. The rate constants are obtained through use of formulas 8 to 11:

$$k_{12} = \frac{C_{11}\lambda_1 + C_{12}\lambda_2}{x_{10}} \qquad 8.$$

$$k_{21} = \frac{k_{12}\ (\lambda_1 + \lambda_2 - k_{12}) - \lambda_1\lambda_2\ (1 - \frac{E}{x_{10}}\ )}{k_{12}} \qquad 9.$$

$$k_{32} = \frac{E\ \lambda_1\lambda_2}{x_{10}k_{21}} \qquad 10.$$

$$k_{23} = \frac{\lambda_1\lambda_2\ \left[1 - \frac{E}{x_{10}}\right]}{k_{12}} - k_{32} \qquad 11.$$

The distribution and rates of transfer of the carrier follow:

$$S_1 = \frac{R_{10}}{x_{10}} \qquad 12.$$

$$\rho_{12} = S_1k_{12} \qquad 13.$$

$$S_2 = \frac{\rho_{12}}{k_{21}} \qquad 14.$$

$$\rho_{23} = S_2k_{23} \qquad 15.$$

$$S_3 = \frac{\rho_{23}}{k_{32}} \qquad 16.$$

Parallel Case.—In the parallel case, access to compartment [2] only is assumed, so equation 6 is obtained by experiment.

$$k_{12} = \frac{-[C_{21}\lambda_1 + C_{22}\lambda_2 - x_{20}(\lambda_1 + \lambda_2)] - \sqrt{[C_{21}\lambda_1 + C_{22}\lambda_2 - x_{20}(\lambda_1 + \lambda_2)]^2 - 4x_{20}\ E\lambda_1\lambda_2}}{2x_{20}} \qquad 17.$$

$$k_{32} = \frac{E\lambda_1\lambda_2}{x_{20}k_{12}} \qquad 18.$$

$$k_{21} = \frac{k_{12}\ (\lambda_1 + \lambda_2 - k_{12} - k_{32}) - \lambda_1\lambda_2 + k_{12}k_{32}}{k_{12} - k_{32}} \qquad 19.$$

$$k_{23} = \frac{C_{21}\lambda_1 + C_{22}\lambda_2}{x_{20}} - k_{21} \qquad 20.$$

$$S_2 = \frac{R_{20}}{x_{20}} \qquad 21.$$

$$\rho_{12} = S_2k_{21} \qquad 22.$$

$$\rho_{23} = S_2k_{23} \qquad 23.$$

$$S_1 = \frac{\rho_{12}}{k_{12}} \qquad 24.$$

$$S_3 = \frac{\rho_{23}}{k_{32}} \qquad 25.$$

Prediction of Tracer Behavior From Known Carrier Kinetics.—This phase of the theory of the behavior of tracers in multicompartment systems has not been emphasized in the literature. It may be of value, however, in devising experiments and in cross-checking the results when the investigator does have experimental access to more than one compartment. In equations 26 to 29 the *k*'s, *xo*'s, and E are assumed to be known. The λ's are the roots of the algebraic quadratic equation, equation 26:

$$(-\lambda)^2 + (k_{12} + k_{21} + k_{23} + k_{32})\ (-\lambda) + (k_{12}k_{23} + k_{12}k_{32} + k_{21}k_{32}) = 0 \qquad 26.$$

Values for ${}_nC_{i1}/E$ for the three compartments and for the three cases depending on whether the tracer is initially in compartment [1], [2], or [3] are presented in Table I. Values for ${}_nC_{i2}/E$ are identical in form to the corresponding ${}_nC_{i1}/E$'s but have λ_2 substituted for λ_1 and vice versa.

TABLE I. EXPLICIT SOLUTIONS FOR THE COEFFICIENTS OF THE EXPONENTIAL TERMS IN EQUATIONS 5, 6, AND 7

	n = 1	n = 2	n = 3
${}_nC_{i1}/E$	$\frac{(k_{12})\ (k_{32}-\lambda_1)\ (\lambda_2)}{(k_{12}-\lambda_1)\ (k_{32})\ (\lambda_1-\lambda_2)}$	$\frac{(k_{32}-\lambda_1)\ (\lambda_2)}{(k_{32})\ (\lambda_1-\lambda_2)}$	$\frac{\lambda_2}{\lambda_1-\lambda_2}$
${}_nC_{21}/E$	$\frac{(k_{32}-\lambda_1)\ (\lambda_2)}{(k_{32})\ (\lambda_1-\lambda_2)}$	$\frac{(k_{12}-\lambda_1)\ (k_{32}-\lambda_1)\ (\lambda_2)}{(k_{12})\ (k_{32})\ (\lambda_1-\lambda_2)}$	$\frac{(k_{12}-\lambda_1)\ (\lambda_2)}{(k_{12})\ (\lambda_1-\lambda_2)}$
${}_nC_{31}/E$	$\frac{\lambda_2}{\lambda_1-\lambda_2}$	$\frac{(k_{12}-\lambda_1)\ (\lambda_2)}{(k_{12})\ (\lambda_1-\lambda_2)}$	$\frac{(k_{32})\ (k_{12}-\lambda_1)\ (\lambda_2)}{(k_{32}-\lambda_1)\ (k_{12})\ (\lambda_1-\lambda_2)}$

The coefficients, ${}_nC_{i2}$, are obtained by substituting λ_2 for λ_1 and λ_1 for λ_2 in the above expressions for ${}_nC_{i1}$.

If an initial distribution is assumed so that some of the tracer is in every compartment, rather than the tracer's being confined to only one, the more general formulas 27, 28, and 29 apply:

$$C_{11} = \frac{k_{12}x_{20} - (k_{12} - \lambda_2)\ x_{10} - \lambda_2\ E}{\lambda_2 - \lambda_1} \qquad 27.$$

$$C_{21} = \frac{k_{21}x_{10} - (k_{21} + k_{23} - \lambda_2)\ x_{20} + k_{23}\ x_{30} - \lambda_2 E}{\lambda_2 - \lambda_1} \qquad 28.$$

$$C_{31} = \frac{k_{32}x_{20} - (k_{32} - \lambda_2)\ x_{30} - \lambda_2 E}{\lambda_2 - \lambda_1} \qquad 29.$$

DISCUSSION

There is an interesting symmetry to be observed among the formulas given in Table I. In particular, if the amount of tracer injected is equal in all cases, the following equalities occur:

$${}_1C_{31} = {}_3C_{11} \qquad {}_1C_{32} = {}_3C_{12} \qquad 30.$$

$${}_1C_{21} = {}_2C_{11} \qquad {}_1C_{22} = {}_2C_{12} \qquad 31.$$

$${}_2C_{31} = {}_3C_{21} \qquad {}_2C_{32} = {}_3C_{22} \qquad 32.$$

This symmetry means that the equation for x_3 when the tracer is initially confined to compartment [1] is identical with the equation for x_1 when the same amount of tracer is initially confined to compartment [3]. The transfer of the tracer across a given boundary or set of boundaries in a system is thus a

function of the kinetics of the entire system, and the appearance equation is independent of the direction of transfer. N. Bauman (personal communication) has shown, using matrix algebra, that the symmetrical result depends upon the condition that $\rho nr = \rho rn$.

It has been implied that the experimenter knows whether a parallel or a series situation is involved in a given experiment. In the event this detail is not known, it may be desired to attempt to make the distinction by assuming first the one case and then the other. In some cases the results will appear reasonable for either assumption, but in others the distinction is possible. If there is access to more than one compartment, comparison of the experimental curves with those predicted from analysis of the other compartment may make the distinction possible. In principle, at least, it also may be possible to make the distinction if the investigator can change the size of one of the compartments; the resulting changes in the rate constants will be different for the two cases.

If units of concentration or of amount are used instead of those of specific activities, the forms of the resulting equations have slight, but important, differences from those of equations 2, 3, and 4. The differences arise from the fact that the amounts and the concentrations of the carrier in the various compartments are not necessarily equal; the equilibrium distribution of the tracer will be proportional to that of the exchangeable carrier. Use of concentration units in the unchanged form of the equations given for specific activities implies the assumption that the concentration of the carrier is the same in all three compartments; similar use of amount units implies that the amounts of the carrier are equal.

The above presentation has neglected an important practical consideration, the effect of experimental error. Relatively small errors in a few of the points on the curve can produce large errors in the constants assigned to fit equations 5, 6, and 7 and these errors are ultimately reflected in the values assigned to the sizes of the compartments and to the rates of transfer. Curve analysis is particularly likely to result in errors in the constants when the equilibrium specific activity is a large fraction of the initial specific activity. In any particular application the effect of experimental error and curve analysis error should be carefully evaluated.

Examples.—The papers of Gellhorn, Merrell, and Rankin[3] and of Cohn and Brues[4] provide good illustrations of the use of analyses based on the parallel and series assumptions, respectively. Berson and Yalow[7] present another example in which the practical difficulties that are encountered in an analysis of a specific problem are discussed. An example of the use of the analysis to help distinguish a parallel from a series configuration of the compartments is presented by Renkin.[8] The results of a three-compartment analysis when a second exponential component appears in the data describing an essentially two-compartment system are presented by Tosteson and Robertson.[9]

SUMMARY

A unified analysis of the behavior of tracers in three-compartment closed systems in the steady state is presented. The relationships needed to deduce

the amounts and rates of exchange of the carrier in and among the compartments of the system from observation of the behavior of the tracer are given explicitly. The converse problem of predicting the behavior of the tracer when the kinetics of the carrier are known is also treated in detail, with particular attention to formulas for the coefficients of the exponential terms. A symmetry in the appearance equations is noted which predicts that when a tracer is introduced into one compartment its appearance in a second is described by the same equation as one obtains when the tracer is introduced into the second compartment and its appearance in the first described. The emphasis is on the practical features of the relationships between the constants which describe the behavior of the tracer and those which characterize the kinetics of the carrier; the formulas required for application of the mathematical basis of the interpretation of the behavior of tracers are given, but the details of their derivations are omitted.

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BLOOD VOLUME AND BODY WATER IN NORMAL HOSPITAL SUBJECTS

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GIBSON and Evans,[1] in their studies almost 20 years ago, found blood volume per unit of body weight to be greater in muscular and in thin individuals than in obese persons. They suggested height rather than body surface area as a standard of reference for blood volume in normal subjects whenever an abnormal relationship existed between height and weight. Perera[2] reached the same conclusion. Gregersen and Nickerson's[3] attempt to narrow the range of blood volume per unit of weight or surface area by grouping their subjects according to Sheldon's body types met with some success at the extremes of the scale only. There are many examples in the literature illustrating the fact that the variation in the amount of relatively avascular fat depots encountered among normal subjects makes body weight and, to a lesser degree, body surface area, poor units of reference for the blood volume except in very lean subjects.

Behnke and associates'[4] concept of the lean body mass (LBM) offers an approach to the problem of predicting the normal blood volume more accurately. The LBM, which represents the actively metabolizing part of the body mass, varies little in its composition throughout adult life[5] and, as pointed out by Behnke,[5] should therefore be considered a more physiologic reference unit for blood volume than the other units of reference.

According to Behnke's[5] definition, the lean body weight (LBW) is the weight of the LBM (specific gravity 1.100). In his densitometric studies on several different normal human populations, he found that the leanest subjects did not exceed a specific gravity of 1.100. As a working hypothesis, therefore, he assumed that this value represented "the over-all density of a lean body mass whose percentage composition is constant from individual to individual." LBW then equals body weight minus weight of depot fat. Excess or depot fat, the substance stored in fat cells, does not in itself utilize oxygen. In contrast, the metabolically active fat tissue has in lean individuals

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about the same blood supply as resting muscle. The LBM, therefore, by definition contains some fat but this is contained in metabolically active fat tissue. Moreover, unlike the "fat free body" determined by chemical analysis of the carcass, the LBM also contains up to 3 per cent of lipid substances, mainly myelin.

LBM as a parameter for blood volume should be valid in subjects ranging from the very lean to the very fat and over a wide range of age. Unless differences can be shown in the composition of the LBM depending on stature or build of the subject, this concept also should hold for the various body types. Furthermore, there is reason to believe that, in terms of LBM, the difference in blood volume between the sexes may be considerably reduced if not abolished, in analogy with the basal metabolic rate in terms of this parameter.[5]

Owing to its constant composition and because water comprises its largest part, the LBM can be computed from the total body water (TBW) in normal individuals of normal hydration.[6, 7] Therefore, TBW should be an equally good reference standard for blood volume as LBW.*

MATERIALS AND METHODS

Forty-two hospital patients (27 men, 15 women) of widely differing weight, stature, build, and fatness were investigated. None were dehydrated or suffering from a condition likely to produce edema, nor receiving drugs known to alter fluid or salt balance. Thirty-five patients were between the ages of 20 and 54 years, 5 were well-developed Puerto Ricans between 17 and 19 years old, while one each was 66 and 72 years old (Subjects 28 and 41, respectively). The majority of patients had recovered from acute respiratory infections. Some were studied before or after minor surgery. One woman was an achondroplastic dwarf (Subject 21) who weighed 75 pounds.

The blood volume (BV), calculated from the sum of the plasma volume (PV) and red cell volume (RCV), was estimated with the patient in the supine position two hours after breakfast in 17 subjects with no evidence of organic disease.† Their ages ranged from 15 to 50 years. Evans Blue (Warner) and autologous P^{32}-labeled red cells, 10 minutes thereafter, were injected to obtain a measure of the plasma volume and red cell mass, respectively. The syringes used for the injection of the test substance were clamped in a holder which prevented withdrawal of the plunger beyond a set distance. This permitted accurate measurement of the volume injected. The volume of distribution of the test substances was obtained by extrapolating from 3 to 4 samples in each case.

The acetone extraction procedure of Chinard[8] was employed for the dye measurement. In the case of P^{32}, 100 λ of blood from a well-mixed 10 ml. sample collected over dry heparin were plated directly on copper planchets, dried slowly, and counted with an end-window G-M tube. The same volume of an appropriately diluted aliquot of the injected tagged red cells was plated, dried, covered with 100 λ of the patient's control plasma, redried, and counted as before to serve as a measure of the injected dose.

In confirmation of the findings of others,[9] the body hematocrit-peripheral hematocrit ratio in these 17 subjects was found to average 0.900 ± 0.027 (0.856 to 0.966). In our series the peripheral hematocrit × 0.96 (to correct for plasma trapping) ranged from 29.7 to 39.3 per cent. As yet no evidence that the ratio is affected by the absolute hematocrit level has been advanced.[9a, c, d] The factor 0.90 then was applied in the remaining patients to calculate BV from the plasma volume (Evans Blue) and the peripheral venous hematocrit.

*Following Behnke and co-workers[7] the LBM is considered to contain 72 per cent water on a weight basis or 72.48 per cent on a volume basis. To convert TBW estimated in liters into LBM the factor 100/72.5 was used in Table I.

†The hematocrit in several patients was below normal. The cause for this, except for Patient 5 who suffered a recent hemorrhage from a peptic ulcer, was unknown.

TABLE I

SUBJECT, SEX, AGE	WEIGHT KG.	HEIGHT CM.	PLASMA VOL. L.	PERIPH. HCRT. × 0.96	RED CELL VOL. L.	BODY HCRT.	BODY HCRT. / PERIPH. HCRT.	BLOOD VOL. L.	TBW L.	LBW KG.	BLOOD VOL. × 100 / BODY WT.	BLOOD VOL. × 100 TBW	EXCESS FAT × 100 / BODY WT.
1. F 18	46.5	146	2.88	36.0	1.14	33.4	0.925	3.42			7.36		
2. F 18	55.5	156	2.19	36.8	1.05	32.4	0.881	3.24			5.85		
3. M 38	68.0	179	3.42	37.4	1.62	32.2	0.861	5.04			7.40		
4. M 34	68.0	167	3.17	40.6	1.79	36.0	0.885	4.96			7.30		
5. F 47	57.0	161	2.85	29.7	1.08	27.4	0.925	3.93			6.90		
6. F 27	51.0	156	2.07	33.0	0.87	29.5	0.898	2.94			5.71		
7. M 15	53.0	175	2.58	38.3	1.38	35.0	0.914	3.96			7.46		
8. M 39	71.0	185	3.71	36.5	1.75	32.0	0.878	5.46	49.0	67.5	7.70	11.12	4.94
9. M 43			3.26	31.2	1.28	28.1	0.908	4.54	40.1	55.4		11.30	
10. F 40	61.5	147	2.22	38.6	1.19	35.0	0.907	3.41	31.2	43.0	5.55	10.90	30.1
11. F 41	95.0	169	2.34	39.5	1.34	36.5	0.925	3.68	35.4	48.9	3.88	10.40	48.5
12. F 54	79.0	170	2.84	33.6	1.21	29.9	0.891	4.05	34.5	47.6	5.13	11.70	39.8
13. M 47	61.5	174	3.44	35.5	1.65	32.4	0.915	5.09	38.4	53.0	8.27	13.22	13.8
14. M 45	62.0	166	3.01	36.6	1.43	32.2	0.881	4.44	42.5	58.6	7.15	10.45	5.5
15. M 50	54.5	178	2.74	39.3	1.48	35.0	0.891	4.22	32.2	44.4	7.76	11.72	18.5
16. M 29	53.0	162	2.55	40.8	1.66	39.4	0.966	4.21	34.9	48.1	7.95	12.10	9.3
17. M 17	45.7	159	2.26	36.4	1.02	31.2	0.856	3.28	29.4	40.6	7.19	11.13	11.1
18. M 51	48.0	164	2.55	38.0				3.88	30.6	42.1	8.08	12.65	12.3
19. M 40	66.6	171	3.66	35.8				5.40	40.6	56.0	7.45	12.40	15.9
20. M 19			2.51	39.5				3.90	38.5	53.1		10.10	
21. F 36	34.0	127	1.72	39.4				2.66	18.0	24.8	7.84	14.75	27.0
22. F 39	60.0	168	2.77	33.5				3.98	39.7	54.7	6.64	10.00	8.8
23. M 36	61.2	169	2.24	37.4				3.38	34.2	47.1	5.53	9.90	23.0
24. F 17	35.4	152	1.45	39.1				2.24	19.2	26.4	6.36	11.68	25.4
25. F 29	53.4	162	2.82	33.8				4.05	32.5	44.9	7.60	12.45	15.9
26. M 38	52.4	161	2.62	41.5				4.18	32.6	45.0	8.00	12.80	14.1
27. M 20	47.6	152	2.18	42.4				3.53	30.1	41.5	7.40	11.70	12.8
28. F 66	102.0	162	2.42	42.1				3.90	37.8	52.1	3.82	10.30	49.0
29. M 18	52.0	169	2.24	44.5				3.74	36.8	51.8	7.20	10.20	0.4
30. F 50	81.0	150	3.03	34.5				4.40	32.6	45.0	5.44	13.50	44.5
31. F 35	67.5	159	2.39	40.2				3.75	32.2	44.4	5.55	11.65	34.2
32. M 23	59.1	167	2.42	41.7				3.88	34.6	47.8	6.56	11.20	19.1
33. M 36	46.6	157	2.40	41.4				3.82	33.7	46.5	8.19	11.30	0.2
34. M 46	87.0	178	3.08	49.0				5.51	49.0	67.5	6.35	11.23	22.4
35. F 35	46.6	154	2.21	34.1				3.19	28.4	39.2	6.85	11.22	15.9
36. M 54	88.2	178	3.89	47.2				6.76	51.5	71.0	7.66	13.10	19.5

37.	F	40	78.0	159	2.50	35.4	3.67	34.1	47.0	4.71	10.75	39.8
38.	M	18	47.1	150	2.04	43.0	3.32	30.0	41.4	7.05	11.05	12.1
39.	F	25	42.3	162	1.94	37.7	2.94	24.0	33.1	6.95	12.23	21.8
40.	M	36	61.6	165	2.64	45.2	4.45	38.8	53.5	7.21	11.48	13.1
41.	M	72	116.0	180	5.12	35.8	7.56	51.1	70.5	6.51	14.75	39.2
42.	M	50	112.2	180	4.20	43.0	6.85	51.9	71.5	6.10	13.20	36.2
43.	M	49	80.0	167	2.57	44.5	4.29	44.0	60.6	5.35	9.75	23.6
44.	F	46	91.5	155	2.97	37.4	4.49	38.8	53.5	4.90	11.55	41.5
45.	F	31	95.6	161	2.26	44.0	3.75	40.1	55.4	3.92	9.35	42.1
46.	M	43	90.0	179	3.30	41.0	5.24	51.0	70.4	5.81	10.03	21.8
47.	M	32	88.5		2.83	41.5	4.53	39.0	53.8	5.12	11.60	39.2
48.	M	49	87.0	160	2.80	46.7	4.84	44.8	61.8	5.61	10.80	29.0
49.	M	32	75.0	186	3.79	41.1	6.02	49.1	67.9	8.04	12.25	9.5
									Mean	6.56	11.55	
									± S.D.	1.2	1.2	
									Coeff. var.	18%	10%	

TBW was estimated from the antipyrine space.[10] A known amount of 5 per cent antipyrine (approximately 20 ml.) was injected intravenously over a period of 4 minutes. The first blood specimen was obtained three to three and one-half hours later, after equilibration presumably was established. Three more blood samples were drawn at intervals of one to one and one-half hours each. The plasma antipyrine was measured by a modification[11] of Brodie's method.[10] The patients were allowed to have a small lunch with no more than 1 glass of liquid one and one-half to two hours after the injection of antipyrine but received nothing by mouth thereafter until the completion of the test. TBW was estimated immediately following the blood volume determination.

RESULTS

The data and derived relations are recorded in Table I. The relationship between BV and body weight is shown in Fig. 1, *A*. There is a positive correlation over the lower and middle weight ranges. However, the slope seems to flatten in the high weight range. Fig. 1, *B* shows a plot of BV against TBW

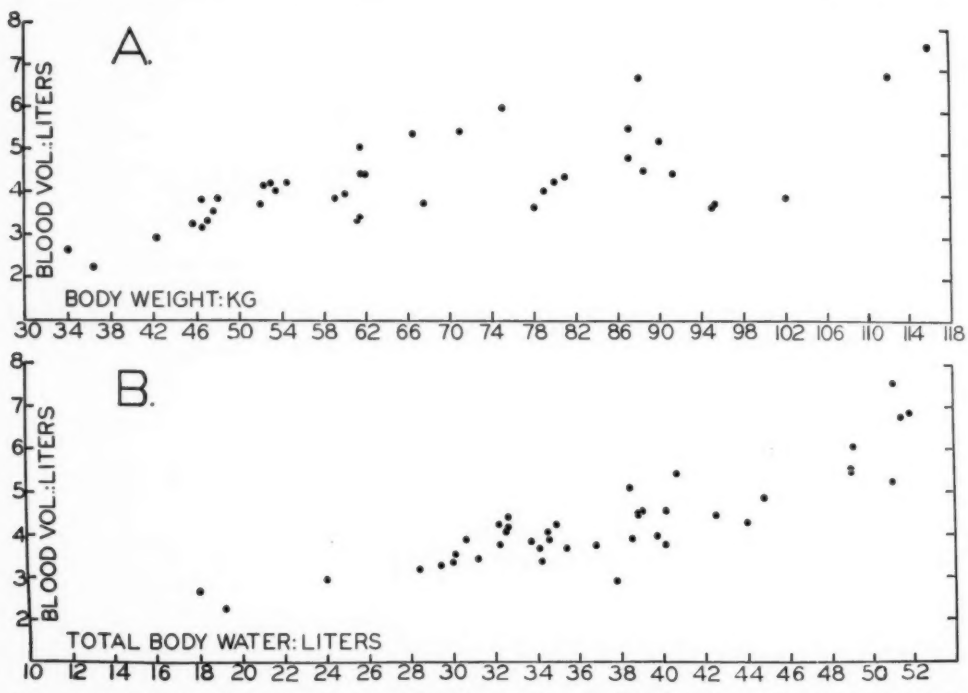

Fig. 1.—*A*, Blood volume plotted against body weight. *B*, Blood volume plotted against total body water.

in the same patients. There is much less scatter than in Fig. 1, *A*, particularly in the higher range. The correlation coefficient for linear regression of BV versus TBW is +0.8856. The straight-line relationship is expressed by the following formula:

Blood volume = 0.1183 TBW − 0.089 (volumes being expressed in liters).

As seen in Table I, the coefficient of variation of BV in terms of TBW is 10 per cent compared with 18 per cent for BV in terms of body weight.

DISCUSSION

The good fit and comparatively small scatter obtained by plotting BV against TBW (Fig. 1, *B*) are the more remarkable in that the measurements on each coordinate carry more than a 5 per cent error. Further, the subjects were both men and women differing in age, weight, stature, habitus, race, and degree of physical activity.* At high TBW values the relatively small number of subjects makes difficult the decision of whether or not the straight-line relationship is replaced by a curve with a slight upward concavity. Alternatively, the three subjects with the highest TBW, who were very muscular and of heavy frame, may have deviated because they had relatively more bone (containing less water than muscle), or relatively larger BV[12] than subjects of more normal build.[3]

The incidence of moderately low hematocrit values observed in this series is typical of the general population of this hospital. The patients come from a low economic stratum and some, no doubt, consume alcohol in larger than average amounts. None of the patients, however, showed signs of malnutrition, overt vitamin deficiency, or clinical or laboratory evidence of hepatic, cardiac, or renal disease. In routine stool examination two of the Puerto Rican patients were found to harbor parasitic worms. The lowered hematocrit values are not likely to invalidate the findings reported here, for the following reasons. BV, and not PV or RCV, has been related to parameters such as body weight or TBW, and BV is presumed to be controlled homeostatically. Consequently, in moderate and even marked chronic anemias regardless of etiology, an increase in plasma volume often completely compensates for a diminished circulating red cell volume.[9e, 13]

It must be remembered that Behnke's concept of an LBM of constant composition and of essentially anhydrous depot fat is an approximation which nevertheless has shown its value as a basis for physiologic measurements when applied to population groups as well as to individual normal subjects whose weight changed. There is reason to believe, however, that moderate weight changes in normal subjects in response to changes in the caloric intake are not merely changes in depot fat.†

*Thirty-six subjects were ambulatory, although several had been up and about for only a few days following a period of bed rest. Four patients were at bed rest and two were wheel-chair patients. Kjelberg, Rudhe and Sjostrand[12] have shown a larger BV (up to 40 per cent) not only in athletes compared with the untrained but also individual increases of 10 to 19 per cent following physical training. Conversely, a decrease (15 per cent) was observed in one patient whose BV was followed serially after one month bed rest for a fracture.

†In their experiments on the density of lost or gained tissue in 7 normal men, Behnke and associates[7] have found a mean value of 0.926 and they presumed this to be the density of (over-all) excess body fat. Keys and Brozek[14] on the other hand quote Jaeckel's value of 0.918 for the specific gravity of subcutaneous fat at 15° C. compared with water at 15° C. Their own figures for extracts of subcutaneous and intra-abdominal human fat obtained at surgery were 0.90074 at 36° C. for the density and 0.9171 at 15° C./15° C. for the specific gravity, values substantially below that of Behnke's obesity tissue. However, their estimate of the mean composition of "total obesity tissue" (density 0.9478) as consisting of 14 per cent extracellular fluid, 62 per cent fat and 25 per cent cellular matter cannot be accepted unreservedly on account of the many assumptions and approximations embodied in the calculation. In support of their argument that the body does not gain or lose fat by itself, Keys and Brozek[14] subjected Pace and Rathbun's data on guinea pigs to a statistical analysis and obtained a positive correlation, which they consider to be significant, between total body fat and the percentage of water in the fat-free body. In their direct analyses Pace and Rathbun[15] found perirenal guinea pig fat to contain some 81.8 per cent fat and 10.25 per cent ± 5.97 water, but such fat depots could perhaps be looked upon in the sense of Behnke as consisting of metabolically active fat tissue plus storage fat and in their former capacity to be entitled to contain water.

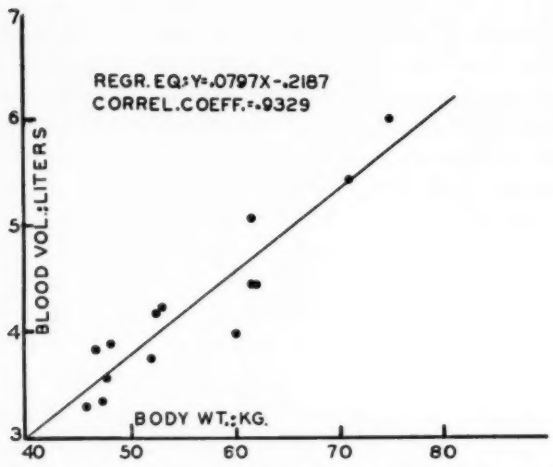

Fig. 2.—Estimated blood volume plotted against body weight in 14 lean subjects. Excess fat was 15 per cent or less of body weight in each subject (excess fat = body weight – 100/72.5 TBW).

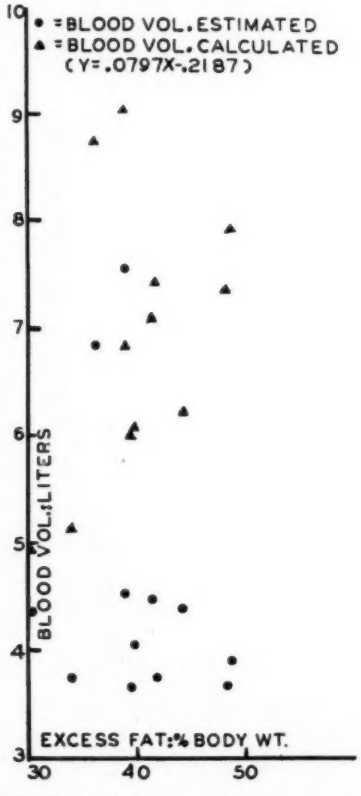

Fig. 3.—Actual and calculated blood volume in 12 obese subjects. Excess fat was 30 per cent or more of body weight in each subject. BV was calculated by substituting individual body weight in the regression equation in Fig. 2.

The present data do not permit conclusions as to the degree of variability of the composition of the LBM in respect to water, nor do they permit differentiation between 'obesity tissue' and essentially anhydrous depot fat. They do indicate, however, that in the absence of edema, dehydration, or marked anemia, TBW fulfills the requirements of a suitable reference unit for blood volume. LBM, estimated densitometrically, can be expected to correlate to a similar degree with BV.

In a recent study, Inkley, Brooks, and Krieger[16] concluded that surface area and weight were superior to TBW (antipyrine) as a means of predicting blood volume. This is not surprising in view of the fact that most of their subjects were lean. Only 14 per cent of their series of 51 subjects could be considered obese (30 per cent or more of the body weight being fat). In the lean person, body weight or surface area probably are better standards of reference for BV than TBW because of the larger error involved in determining TBW.

Most workers in the field agree that mean values and coefficients of variations for absolute blood volume or for its subdivisions have little meaning in view of the variable composition of the populations sampled regarding build, stature, sex, age, race, etc. For the same reason, the mean blood volume of a series of subjects and its coefficient of variation based on body weight, body surface area, or height can be of only limited value. Furthermore, disregard of hematocrit corrections for plasma trapping or for the body-peripheral hematocrit ratio where only one component of the blood volume was measured can lead to significant errors. In the estimation of plasma volume with Evans Blue dye the use of a single ten-minute plasma, although frequently accurate enough for clinical work, can lead to serious errors. Whenever possible dye extraction should be employed on both the unknown sample and the dye-plasma standards, respectively.

Huff and Feller[17] related measured RCV (chromium^{51} method) and calculated blood volume (no correction for trapping or the hematocrit ratio) to body density in a population of 42 normal men and 20 women. About one-half of the subjects were obese, fat constituting 30 per cent or more of their weight. The authors obtained a correlation coefficient of 0.62 for 'blood volume per kg. body weight' versus 'body density.' Subtracting from body weight the amount of total fat calculated from the Rathbun and Pace equation[18] and the Keys and Brozek equation,[14] respectively, they obtained two values for the LBM (actually the 'fat-free body'). The correlation coefficients for BV against the two LBM values were 0.719 and 0.840, respectively, for the whole population of 62 subjects. They also attempted to obtain an estimate of the relative blood volume contained in "obesity" tissue by extrapolating on the plasma volume per kilogram of body weight versus density plot to a value of 0.948, considered by Keys and Brozek[14] to be the density of obesity tissue. Such an extrapolation is doubtful in view of the fact that the observed densities lay between about 0.995 and 1.09 and the gap to be bridged by extrapolation was considerable. The authors then investigated the possibility of

improving predictability of blood volume and its subdivisions by employing multivariant regression equations and including parameters other than the customary weight or surface area. Correlation coefficients were given for both sexes separately between BV, RCV, or PV on the one hand and LBM or fat weight on the other. These simple correlation coefficients were then compared with those obtained when LBM and fat weight were considered together as variables. The trivariant coefficients were found to be significantly larger than the bivariant ones. There is no indication, however, whether in their multivariant analyses the authors have taken into account the interrelation between LBM and fat, quantities estimated from the same measurements, namely, weight and density. Neglect of the interdependence of the two variables would make the final correlation coefficient inordinately large.

Taylor, Brozek, and Keys' findings[19] in a related field deserve mention at this point. These authors found that the basal cardiac output of normal human subjects could be predicted most accurately from the LBM and that only a small advantage in predictability was obtained by using multiple regression equations embodying both LBM and fat weight.

In abnormal states such as edema, LBM as unit of reference for BV should be computed from sources other than TBW, perhaps from the basal metabolic rate[20] or creatinine excretion.[21] Moreover, the heart volume may well be an important determining factor for BV. Kjelberg and his colleagues[22] have shown a high correlation (corr. coeff. + 0.90) of BV with heart volume in normal subjects. In congestive heart failure (enlargement of heart and liver, edema, and possibly malnutrition) the LBM calculated by whatever known method may not be a valid standard of reference for blood volume.

SUMMARY

In 17 subjects (15 to 50 years) with no evidence of disease, the body hematocrit–peripheral hematocrit ratio was 0.900 ± 0.027 (range 0.856 to 0.966). The factor 0.90 was subsequently applied in the computation of the blood volume from peripheral hematocrit and the estimated plasma volume.

Total body water (TBW) was determined by the antipyrine method and the blood volume calculated as above in 42 patients (27 men, 15 women) of widely differing weight, stature, build, and fatness. None were dehydrated or suffering from a condition likely to produce edema. A straight-line relationship between blood volume and total body water was apparent with a correlation coefficient of 0.8856.

The concept of lean body mass (LBM, Behnke) is discussed. By virtue of the constant relationship to total body water, LBM would appear to be equally good as a standard of reference for blood volume and better than body weight or surface area, particularly in obese subjects.

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THE INFLUENCE OF IONIZING RADIATION ON THE BODY COMPOSITION OF PATIENTS WITH MALIGNANT LYMPHOMA

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THE effects of ionizing radiation upon the mammalian economy have been studied extensively in recent years. However, most of the investigations reported in the available literature have used *small* animals as their experimental subjects. Furthermore, they are usually "acute studies" employing radiation doses which result in the death of the animal. Another striking feature is the large number of conflicting results and divergent conclusions, some of which, at least, may be explained by species differences and lack of standardization of experimental conditions such as x-ray dosage. Thus, Chamberlain recently called attention to the need for basic research on the effects of ionizing radiation in *large* animals, so that the mechanisms underlying radiation injury could be better understood and more readily extrapolated.[1]

Since, ultimately, all animal data must be interpreted in terms of the human organism it was felt that patients undergoing radiation therapy for malignant tumors could serve as experimental subjects to provide direct information. This paper concerns itself with data which show the influence of ionizing radiation upon the total body composition of such patients. Through serial and simultaneous determinations of the total body water and the nitrogen balance, the shifts induced in the lean body tissue and fat are outlined.

PROCEDURE

Four patients with malignant lymphoma were studied on the metabolic research ward for 60 to 126 days. The study was divided into a control, radiation, and postradiation phase. The control phase lasted a minimum of four metabolic periods†; the treatment phase was three to four periods in duration and the patients were then observed for two to three additional metabolic periods following the completion of radiotherapy.

During the entire study the dietary offerings were kept constant. Two of the patients (C. M. and G. E.) were maintained on a "normal" diet. This contained a daily average of 1,830 calories (64 Gm. protein, 62 Gm. fat, and 254 Gm. carbohydrate). The other two patients (I. T. and S. C.) received a diet adequate in calories, but low in proteins and deficient in B-vitamins and certain minerals, in an attempt to gauge what role the nutritional status may have upon radiation sensitivity. This latter diet was

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†A metabolic period consists of six days.

essentially a Kempner rice diet[2] modified only by avoiding those fruits which had a high B-vitamin content and giving no vitamin and iron supplement. The daily allotment contained 2,550 calories (26 Gm. protein, 2 Gm. fat, and 608 Gm. carbohydrate). Water was permitted ad libitum in both the "normal" and "restricted" diets.

The patients were weighed daily immediately following the early morning urine collection. They were allowed to ambulate on the metabolic ward as their condition permitted, but their activities were limited to light work (such as sewing).

Serial simultaneous determinations of various body constituents including the body composition were performed before, during, and after exposure to ionizing radiation. Nitrogen, phosphorus, calcium, potassium, sodium, and water balances were measured throughout the entire study. Urinary uric acid, creatine, and creatinine determinations were performed daily. Clinical and laboratory data, including roentgenograms obtained at frequent intervals, supplemented the study.

Aliquots of every food served were analyzed at the end of each metabolic period for nitrogen, calcium, phosphorus, sodium, and potassium. Aliquots of the pooled urine and pooled feces of each six-day period were similarly analyzed. The metabolic balances were determined for each six-day period by comparing the intake and the total excretion.

Since in each of the four patients the metabolic balances, the urinary uric acid, and creatinine excretions, as well as the various blood constituents, remained essentially constant (at least in the last two periods of the control phase), it seems justified to assume that by that time all the patients had achieved a "steady metabolic state." The values for the body composition obtained during these last two control periods served as the base line for the values obtained during the radiotherapy and postradiation phases (Tables I-IV).

The radiation regimen and the clinical data were described in a previous publication.[3]

After the postradiation phase was completed the patients were discharged from the metabolic ward. Those patients who were maintained on the "restricted" diet were then restudied 35 to 39 days after being placed on a diet comparable to the "normal" diet given on the metabolic ward (Table II).

MATERIALS AND METHODS

The "heavy water space" (total body water) was measured as the two-hour volume of distribution of intravenously injected deuterium oxide, as previously described.[3] The chemical methods employed for analysis of the various constituents, such as nitrogen in blood, urine, or ashed samples of diet or stool, also were described in a previous publication.[4]

The calculations in this paper are based on the following relationships[5-8]:

Weight − total body water = total body solids (A)
Lean body solids + body fat = total body solids (B)
Lean body solids + total body water = lean body mass (C)
Weight − lean body mass = body fat (D)
Total body water ÷ 0.732 = lean body mass (E)

Normally, where the water content is known the per cent of fat is derived as:

$$\text{Per cent fat} = 100 - \frac{\%\ \text{water}}{0.732} \quad \text{(F)}$$

Similarly, the specific gravity may be calculated from:

$$\text{Per cent fat} = 100\ \left(\frac{5.548}{\text{Sp. Gr.}} - 5.044\right) \quad \text{(G)}$$

Where nitrogen balance is measured:

$$\Delta\ \text{N balance} \times 30 = \Delta\ \text{lean body mass} \quad \text{(H)}$$

Since lean tissue is 73.2 per cent water, it follows that:

$$\Delta\ \text{N balance} \times 21.9 = \Delta\ \text{lean body water} \quad \text{(I)}$$
$$\Delta\ \text{N balance} \times 8.1 = \Delta\ \text{lean body solids} \quad \text{(J)}$$

RESULTS

A. Based on Deuterium Oxide Space.—

"Normal" diet: (The pertinent experimental data are presented in Table I and Fig. 1.)

CONTROL PHASE: During this phase the various components listed were essentially within the limits accepted as normal in both patients.

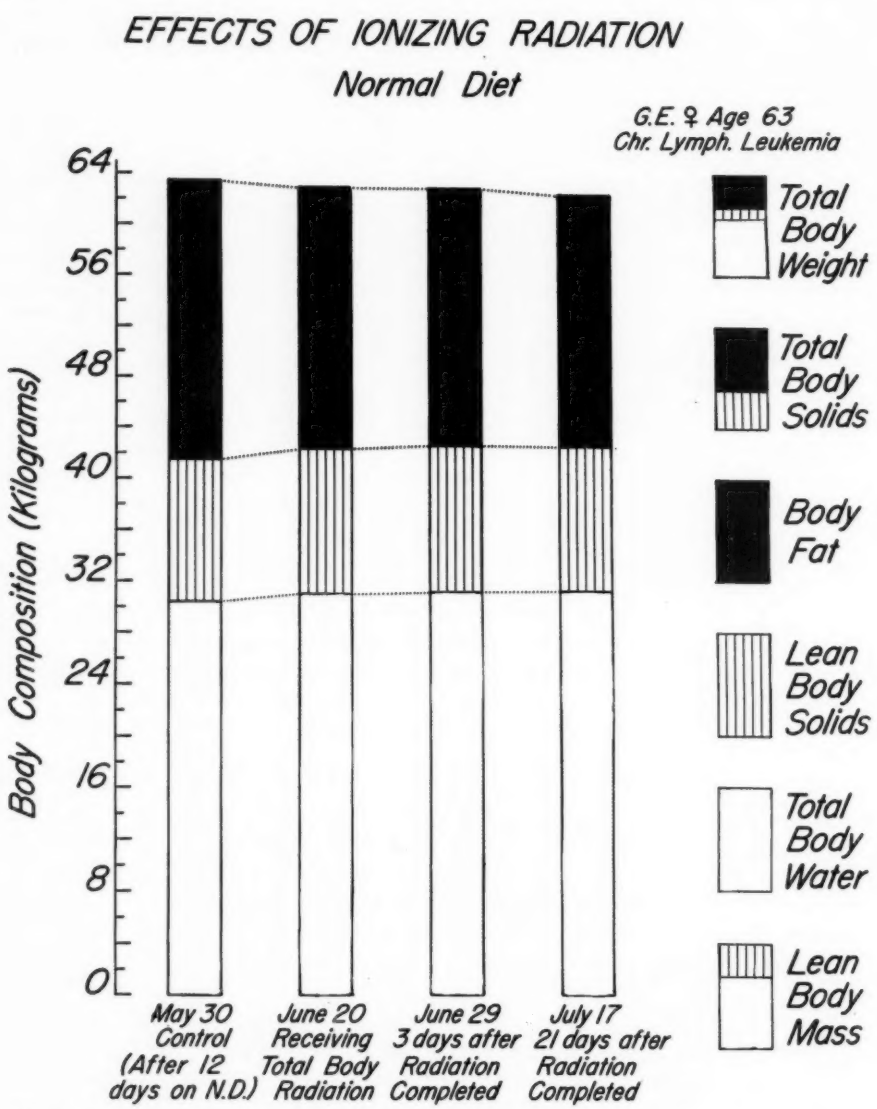

Fig. 1.—Effects of ionizing radiation on the body composition of a patient with malignant lymphoma when maintained on a "normal" diet.

RADIATION PHASE: The lean body components did not show any striking changes under the influence of radiation. There was a mild reduction in the

weight of both patients which was entirely accounted for by a concomitant diminution in the body fat (1.6 kilograms in Patient C. M.; 1.8 kilograms in Patient G. E.). The body specific gravity rose proportionately.

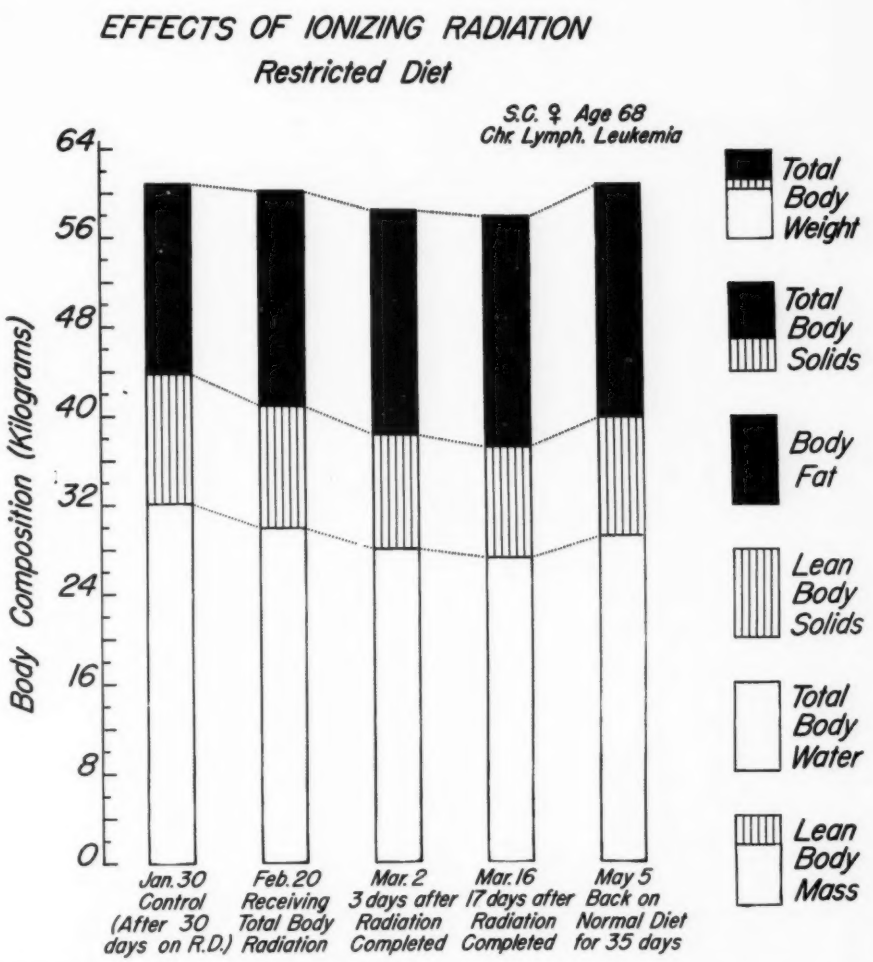

Fig. 2.—Effects of ionizing radiation on the body composition of a patient with malignant lymphoma when maintained on a "restricted" diet.

POSTRADIATION PHASE: Restudy of the body composition 21 days after the last dose of total body x-radiation had been given showed an additional mild reduction in weight, again due entirely to fat loss. The total fat loss between the first and last study was 3.1 kilograms in Patient C. M., and 2.2 kilograms in Patient G. E.

"Restricted" diet: (The pertinent experimental data are presented in Table II and Fig. 2.)

CONTROL PHASE: Here, as in the patients receiving a "normal" diet the various components of the body composition listed were within normal limits.

TABLE I. EFFECTS OF IONIZING RADIATION ON THE BODY COMPOSITION* OF PATIENTS WITH MALIGNANT LYMPHOMA WHEN MAINTAINED ON A "NORMAL" DIET

PATIENT, AGE, SEX, DIAGNOSIS	STATUS AND DATE OF MEASUREMENT	WT. (KG.)	SURF. AREA (M^2)	TOTAL BODY SOLIDS			FAT			LEAN BODY SOLIDS			TOTAL BODY WATER			LEAN BODY MASS			BODY SPECIFIC GRAVITY
				KG.	% B.W.	KG./M^2	KG.	% B.W.	KG./M^2	KG.	% B.W.	KG./M^2	L.	% B.W.	L./M^2	KG.	% B.W.	KG./M^2	
	Control (performed after 12 days on "normal" diet) (1/6/53)	67.2	1.76	29.0	43.1	16.5	15.0	22.3	8.52	14.0	20.8	7.98	38.2	56.9	21.8	52.2	77.7	29.8	1.053
C. M.	Receiving T.B.R.† (10r *ant.* and 15r *post.* to date) (1/28/53)	66.1	1.75	27.8	42.0	15.9	13.8	20.8	7.88	14.0	21.2	8.03	38.3	58.0	22.0	52.3	79.2	30.0	1.056
43, Male	3 days after conclusion of T.B.R. (25r *ant.*; 30r *post.*) (2/5/53)	65.6	1.73	27.4	41.7	15.8	13.4	20.4	7.71	14.0	21.3	8.08	38.2	58.3	22.1	52.2	79.6	30.2	1.057
Chronic lymphatic leukemia	21 days after conclusion of T.B.R. (2/23/53)	64.5	1.72	26.0	40.3	15.1	11.9	18.4	6.89	14.1	21.9	8.18	38.5	59.7	22.3	52.6	81.6	30.5	1.061
	Changes between first and last study	−2.7	−0.04	−3.0	−2.8	−1.4	−3.1	−3.9	−1.63	+0.1	+1.1	+0.20	+0.3	+2.8	+0.5	+0.4	+3.9	+0.7	+0.008
	Control (performed after 12 days on "normal" diet) (5/30/53)	63.5	1.64	33.1	52.1	20.2	22.0	34.6	13.4	11.1	17.5	6.78	30.4	47.9	18.5	41.5	65.4	25.3	1.029
G. E.	Receiving T.B.R. (25r *ant.* and 25r *post.* to date) (6/20/53)	63.0	1.63	32.0	50.8	19.6	20.6	32.8	12.6	11.4	18.0	6.95	31.0	49.2	19.0	42.4	67.2	25.9	1.033
63, Female	3 days after conclusion of T.B.R. (50r *ant.*; 50r *post.*) 6/29/53)	62.9	1.63	31.7	50.3	19.4	20.2	32.1	12.4	11.5	18.2	7.02	31.2	49.7	19.2	42.7	67.9	26.2	1.034
Chronic lymphatic leukemia	21 days after conclusion of T.B.R. (7/17/53)	62.4	1.63	31.2	50.0	19.2	19.8	31.8	12.2	11.4	18.2	7.01	31.2	50.0	19.2	42.6	68.2	26.2	1.035
	Changes between first and last study	−1.1	−0.01	−1.9	−2.1	−1.0	−2.2	−2.8	−1.2	+0.3	+0.7	+0.23	+0.8	+2.1	+0.7	+1.1	+2.8	+0.9	+0.006

*Based on D_2O space (D_2O space ÷ 0.732 = lean body mass). †T.B.R. = Total body x-radiation.

TABLE II. EFFECTS OF IONIZING RADIATION ON THE BODY COMPOSITION* OF PATIENTS WITH MALIGNANT LYMPHOMA WHEN MAINTAINED

TABLE II. EFFECTS OF IONIZING RADIATION ON THE BODY COMPOSITION* OF PATIENTS WITH MALIGNANT LYMPHOMA WHEN MAINTAINED ON A "RESTRICTED" DIET

PATIENT, AGE, SEX, DIAGNOSIS	STATUS AND DATE OF MEASUREMENT	WT. (KG.)	SURF. AREA (M^2)	TOTAL BODY SOLIDS			FAT			LEAN BODY SOLIDS			TOTAL BODY WATER			LEAN BODY MASS			BODY SPECIFIC GRAVITY
				KG.	% B.W.	KG./M^2	KG.	% B.W.	KG./M^2	KG.	% B.W.	KG./M^2	L.	% B.W.	KG./M^2	KG.	% B.W.	L./M^2	
	Control (performed after 78 days on "restricted" diet) (4/6/53)	62.7	1.64	27.4	43.7	16.7	14.5	23.1	8.83	12.9	20.6	7.86	35.3	56.3	21.5	48.2	76.9	29.4	1.052
I. T.	Receiving Co^{60} teletherapy (2,574r† to date) (5/2/53)	60.4	1.62	27.0	44.8	16.7	14.8	24.5	9.16	12.2	20.3	7.55	33.4	55.2	20.6	45.6	75.5	28.2	1.049
43, Male	1 day after conclusion of Co^{60} (total of 4,212r)† (5/12/53)	59.8	1.61	26.9	45.0	16.7	14.9	24.9	9.26	12.0	20.1	7.48	32.9	55.0	20.4	44.9	75.1	27.9	1.048
Reticulum cell lymphosarcoma	13 days after conclusion of Co^{60} (5/24/53)	59.2	1.60	26.5	44.7	16.5	14.5	24.5	9.04	12.0	20.2	7.46	32.7	55.3	20.4	44.7	75.5	27.9	1.049
	Changes between first and last study	−3.5	−0.04	−0.9	+1.0	−0.2	0	+1.4	+0.21	−0.9	−0.4	−0.40	−2.6	−1.0	−1.1	−3.5	−1.4	−1.5	−0.003
	Back on "normal" diet for 39 days (7/15/53)	62.0	1.64	27.6	44.5	16.8	15.0	24.2	9.15	12.6	20.3	7.68	34.4	55.5	21.0	47.0	75.8	28.7	1.050
	Control (performed after 30 days on "restricted" diet) (1/30/53)	60.9	1.56	28.8	47.3	18.4	17.0	28.0	10.9	11.8	19.3	7.53	32.1	52.7	20.6	43.9	72.0	28.1	1.042
S. C.	Receiving T.B.R.‡ (25r *ant.* and 25r *post.* to date) (2/20/53)	60.4	1.56	30.4	50.3	19.6	19.4	32.1	12.5	11.0	18.2	7.07	30.0	49.7	19.3	41.0	67.9	26.4	1.034
68, Female	3 days after conclusion of T.B.R. (50r *ant.*; 50r *post.*) (3/2/53)	58.7	1.54	30.6	52.1	19.9	20.3	34.6	13.2	10.3	17.5	6.69	28.1	47.9	18.3	38.4	65.4	25.0	1.029
Chronic lymphatic leukemia	17 days after conclusion of T.B.R. (3/16/53)	58.2	1.53	30.8	52.9	20.1	20.8	35.7	13.6	10.0	17.2	6.54	27.4	47.1	17.9	37.4	64.3	24.4	1.027
	Changes between first and last study	−2.7	−0.03	+2.0	+5.6	+1.7	+3.8	+7.7	+2.7	−1.8	−2.1	−0.99	−4.7	−5.6	−2.7	−6.5	−7.7	−3.7	−0.015
	Back on "normal" diet for 35 days (5/5/53)	60.9	1.56	31.6	51.9	20.2	20.9	34.3	13.4	10.7	17.6	6.86	29.3	48.1	18.8	40.0	65.7	25.6	1.030

Based on D_2O space (D_2O space ÷ 0.732 = lean body mass. †Tumor dose. ‡T.B.R. = Total body x-radiation.

TABLE III. EFFECTS OF IONIZING RADIATION ON THE BODY COMPOSITION* OF

PATIENT, AGE, SEX, DIAGNOSIS	STATUS AND DATE OF MEASUREMENT (1953)	Δ WT. (GM.)	NITROGEN BALANCE MG./DAY	Δ TOTAL BODY SOLIDS		Δ FAT	
				GM.	GM./DAY	GM.	GM./DAY
	Control (1/6–1/18)	–349	+830	–567	–47.3	–648	–54.0
C. M.	T.B.R.† (1/18–2/5)	–1,329	–827	–1,003	–55.7	–882	–49.0
43, Male	Post. T.B.R. (2/5–2/23)	–949	+1,010	–1,347	–74.8	–1,494	–83.0
Chronic lymphatic leukemia	Net changes during entire study (1/6–2/23)	–2,627	+276	–2,917	–60.8	–3,024	–63.0
	Control (5/30–6/11)	+120	+2,458	–526	–43.8	–765	–63.7
G. E.	T.B.R. (6/11–6/29)	–821	+433	–992	–55.1	–1,055	–58.6
63, Female	Post. T.B.R. (6/29–7/17)	–409	–156	–348	–19.4	–325	–18.1
Chronic lymphatic leukemia	Net changes during entire study (5/30–7/17)	–1,110	+718	–1,866	–38.9	–2,145	–44.7

*Based on nitrogen balance (Δ nitrogen balance ×30 = Δ lean body mass).
†T.B.R. = Total body x-radiation.

RADIATION PHASE: In sharp contrast to the patients receiving a "normal" diet both patients on the "restricted" diet showed a significant decrease in their lean body components (3.3 kilograms lean body mass in Patient I. T.; 5.5 kilograms lean body mass in Patient S. C.). Also in sharp contrast was the fact that despite the losses in the lean body components both patients on the "restricted" diet showed increases in the body fat (0.4 kilogram in Patient I. T.; 3.3 kilograms in Patient S. C.). This resulted in a dilution of the total weight loss during this phase. The combination of loss in lean body mass and gain in fat caused a lowering of the body specific gravity in both patients.

POSTRADIATION PHASE: The body composition was remeasured 13 days (I. T.) and 17 days (S. C.) after the conclusion of radiation. The lean body components remained markedly reduced as compared to the control study; the over-all loss in lean body mass being 3.5 kilograms in Patient I. T. and 6.5 kilograms in Patient S. C. During this phase Patient I. T. lost 0.4 kilogram fat so that his total weight loss during the entire period of study was wholly accountable by his loss in lean body mass. Patient S. C. on the other hand gained an additional 0.5 kilogram fat (total body fat gain being 3.8 kilograms). This 3.8 kilograms fat gain offset somewhat the 6.5 kilograms loss of lean body mass so that the total weight loss was only 2.7 kilograms.

Restudy of Patients S. C. and I. T., 35 and 39 days, respectively, after being replaced on a "normal" diet: The body fat did not show any striking alterations. Both patients did, however, show a significant increase in their lean body components (2.3 kilograms lean body mass in Patient I. T.; 2.6 kilograms lean body mass in Patient S. C.). This increase, although substantial, did not raise these components to the levels found in the control study.

PATIENTS WITH MALIGNANT LYMPHOMA WHEN MAINTAINED ON A "NORMAL" DIET

△ LEAN BODY SOLIDS		△ LEAN BODY WATER		△ LEAN BODY MASS		INTAKE	CALS./DAY	APPARENT DAILY CALORIC REQIREMENT
GM.	GM./DAY	ML.	ML./DAY	GM.	GM./DAY	CALS./DAY	FROM FAT	
+81	+6.7	+218	+18.2	+299	+24.9	1,824	+486	2,310
−121	−6.7	−326	−18.1	−477	−24.8	1,830	+441	2,271
+147	+8.2	+398	+22.1	+545	+30.3	1,829	+747	2,576
+107	+2.2	+290	+6.0	+397	+8.3	1,828	+567	2,395
+239	+19.9	+646	+53.8	+885	+73.7	1,830	+574	2,404
+63	+3.5	+171	+9.5	+234	+13.0	1,831	+527	2,358
−23	−1.3	−61	−3.4	−84	−4.7	1,821	+163	1,984
+279	+5.8	+756	+15.8	+1,035	+21.6	1,827	+402	2,229

B. Based on Changes in the Nitrogen Balance.—

"Normal" diet: (The pertinent experimental data are presented in Table III.)

The changes observed in the lean body components and fat were essentially identical with those seen when heavy water was used as the reference measurement. There was a mild reduction in weight due entirely to a diminution in the body fat. The nitrogen balance showed a tendency toward negativity under the influence of the ionizing radiation, but the over-all changes were not striking.

"Restricted" diet: (The pertinent experimental data are presented in Table IV.)

Here too the changes observed in the body composition were essentially identical with those seen when deuterium oxide was used as the reference measurement. Both patients sustained significant losses in the lean body components as the result of being exposed to ionizing radiation ($>$ 5 kilograms lean body mass in Patient S. C.). Correspondingly, the nitrogen balances showed marked negativity during this phase ($>$ 9,500 mg. per day in Patient S. C.).

In sharp contrast to the losses sustained in the lean body components was the gain in fat shown by both patients during the radiation phase ($\overline{ca}$ 2 kilograms in Patient S. C.). The apparent daily caloric requirement during this phase was remarkably low (1,137 Calories in Patient I. T.; 1,172 Calories in Patient S. C.).

DISCUSSION

The increased nitrogen excretion observed following exposure to ionizing radiation is in accord with the findings of numerous other investigators.[9-15]

TABLE IV. EFFECTS OF IONIZING RADIATION ON THE BODY COMPOSITION* OF

PATIENT, AGE, SEX, DIAGNOSIS	STATUS AND DATE OF MEASUREMENT (1953)	Δ WT. (GM.)	NITROGEN BALANCE (MG./DAY)	Δ TOTAL BODY SOLIDS		Δ FAT	
				GM.	GM./DAY	GM.	GM./DAY
	Control (4/6–4/18)	–420	–1,442	–41	–3.4	+99	+8.3
I. T.	Cobalt^{60} teletherapy (4/18–5/12)	–2,450	–3,733	–488	–20.3	+238	+9.9
43, Male	Post. Cobalt^{60} (5/12–5/24)	–630	–639	–462	–38.5	–400	–33.3
Reticulum cell lympho-sarcoma	Net changes during entire study (4/6–5/24)	–3,500	–2,387	–991	–20.6	–63	–1.3
	Control (1/30–2/11)	+1,248	0	+1,248	+104.0	+1,248	+104.0
S. C.	T.B.R.† (2/11–3/1)	–3,242	–9,648	+561	+31.2	+1,968	+109.3
68, Female	Post. T.B.R. (3/1–3/16)	–731	–2,733	+167	+11.2	+499	+33.3
Chronic lymphatic leukemia	Net changes during entire study (1/30–3/16)	–2,725	–4,770	+1,976	+43.9	+3,715	+82.6

*Based on nitrogen balance (Δ nitrogen balance ×30 = Δ lean body mass).
†T.B.R. = Total body x-radiation.

That the resulting negative nitrogen balance is due, at least in good measure, to tissue destruction is attested to by the fact that the greatest negativity is observed in those patients who also showed the greatest regression of their tumor masses.

The calculations employed to measure the shifts in body composition bear some comment. They are based on the observation that the lean tissue of the "normal" adult has a constant water and nitrogen content. Now, although these relationships may not be precise in the face of changing lean tissue composition (disease states, exposure to ionizing radiation, and so on) they do offer an opportunity to at least gain a first order of approximation of the changes taking place in the body composition under these conditions. Thus, the trends in all the cases studied were consistent and the changes were of the proper order of magnitude. Furthermore, the excellent agreement of the data, derived from two entirely independent experimental methods of measurement, lends additional credence to the validity of the findings.

Both patients on the "restricted" diet sustained substantial losses in lean body solids and total body water. These losses paralleled the losses in lean body mass and also correlated well with the markedly negative nitrogen balances. It is important to emphasize that by following the body weight changes only, one would not have obtained an accurate index of the quantity of "active cellular mass" being lost by the patients, since they were gaining body fat at the very time that they were losing considerable lean body mass, thereby diluting the actual "protoplasmic" losses. Thus, during the course of the study, Patient S. C. lost 6.5 kilograms lean body mass, but gained 3.8 kilograms fat so that the total weight loss was only 2.7 kilograms. Of additional interest is

PATIENTS WITH MALIGNANT LYMPHOMA WHEN MAINTAINED ON A "RESTRICTED" DIET

△ LEAN BODY SOLIDS		△ LEAN BODY WATER		△ LEAN BODY MASS		INTAKE	CALS./DAY	APPARENT DAILY CALORIC REQUIREMENT
GM.	GM./DAY	ML.	ML./DAY	GM.	GM./DAY	CALS./DAY	FROM FAT	
−140	−11.7	−379	−31.6	−519	−43.3	1,263	−74	1,189
−726	−30.2	−1,962	−81.8	−2,688	−112.0	1,226	−89	1,137
−62	−5.2	−168	−14.0	−230	−19.2	1,676	+300	1,976
−928	−19.3	−2,509	−52.3	−3,437	−71.6	1,348	+12	1,360
0	0	0	0	0	0	2,625	−936	1,689
−1,407	−78.1	−3,803	−211.3	−5,210	−289.4	2,156	−984	1,172
−332	−22.1	−898	−59.9	−1,230	−82.0	1,882	−299	1,583
−1,739	−38.6	−4,701	−104.5	−6,440	−143.1	2,190	−743	1,447

the fact that during the period of greatest nitrogen negativity the caloric requirements in both patients were $< 1{,}200$ Calories per day. It would appear, then, that with adequate caloric intake tissue destruction may take place without corresponding changes in body weight, because of concomitant increases in body fat.

The gain in body fat during a time when marked catabolic changes were taking place may be at least partially explained by assuming that some of the protein liberated underwent gluconeogenesis, with ultimate conversion into fat. Thus, several investigators have reported a hyperglycemia and liver glycogen accumulation in their subjects following exposure to ionizing radiation.[16-18] Ord and Stocken[19] state that this glycogen accumulation is probably not due to a block in the oxidation of the substance, but that it is synthesized from C_2 fragments resulting from the tissue breakdown caused by direct radiation damage. Bacq and associates[20] observed alterations in the fat levels and the whole body fat of irradiated rats, and suggested increased synthesis of fat as a possible explanation.

Data have been presented which suggest that the mechanism of the gluconeogenesis is through the pituitary-adrenal system.[16, 18] Since exposure to ionizing radiation does cause increased pituitary-adrenal activity[3] the increased gluconeogenesis may represent part of the body's response to radiation insult.

The striking difference between the patients on the "restricted" and those on the "normal" diet in the degree of clinical as well as laboratory response to radiation suggests that the "restricted" diet may have in some way made the tissues more radiosensitive, resulting in an enhancement of the radiation effects. Thus, Jennings[21] found that protein depletion in rats prior to

irradiation made these animals more sensitive to total body irradiation, although post-mortem examination did not reveal any difference in the x-ray lesions from those of the controls. Since adrenal activity is increased following radiation, these findings also fit in with the observations of Eliel and Heaney[22] who reported that administration of cortisone to a patient with chronic lymphatic leukemia maintained on a protein-free diet was associated with rapid regression of enlarged nodes, and elevations in the excretion of nitrogen, phosphorus, and potassium.

The data presented in this paper support the contention made in a previous publication[3] that following exposure to ionizing radiation there occurs a cellular injury leading to a loss of intracellular constituents. The degree of injury depends upon the amount of radiation exposure and the radiosensitivity of the tissue. If the radiation dose is too large or the tissue very radiosensitive, irreversible cell destruction may occur resulting in the permanent escape of the intracellular constituents (water, nitrogen, phosphorus, potassium, and so on) to the extracellular environment and/or the external world, with the appearance of the end products of tissue catabolism in the excreta. Thus, in this study Patient C. M. who received the least radiation also showed the least changes in the compartments of his body composition, as well as the smallest clinical and metabolic response. On the other hand, both patients maintained on the "restricted" diet showed a very marked clinical and catabolic response to the ionizing radiation. They showed striking regression of their lesions and marked negativity in their nitrogen, phosphorus, and potassium balances, as well as marked increases in uric acid excretion.[23] They also showed a very significant reduction in lean body mass.

It may be that this reduction in the lean body mass could serve as a sensitive indicator of the amount of irreversible cellular destruction taking place.

SUMMARY

The effects of ionizing radiation on the body composition of four patients with malignant lymphomas were studied under controlled metabolic conditions. Throughout the entire study two of the patients were maintained on a "normal" diet; two received a diet adequate in calories, low in protein and deficient in B-vitamins and certain minerals (modified Kempner rice diet). The alterations occurring in the lean tissues and fat of the body were ascertained with the aid of serial simultaneous determinations of the total body water and nitrogen balance. There was excellent agreement of the data derived from these two independent methods of measurement.

In sharp contrast to the patients receiving a "normal" diet the patients on the "restricted" diet showed a significant reduction in their lean body components, and a markedly negative nitrogen balance following exposure to ionizing radiation. Their body fat, however, increased. The implications of these findings are discussed.

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HEMODYNAMIC DATA DURING REST AND EXERCISE IN PATIENTS WITH MITRAL VALVE DISEASE IN RELATION TO THE DIFFERENTIATION OF STENOSIS AND INSUFFICIENCY FROM THE PULMONARY ARTERY WEDGE PRESSURE PULSE

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THE pulmonary artery wedge pressure pulse in man has been demonstrated to be a moderately damped but reasonably accurate representation of the left atrial pulse, in pressure and usually in contour.[1, 2]

A characteristic increase in the magnitude of the systolic (V) wave of the pulmonary artery wedge pressure pulse has been described in mitral insufficiency, and attributed to the regurgitation of blood into the left atrium through the incompetent mitral valve during ventricular systole.[3-5] However, the magnitude of the V wave has not in itself proved consistently useful in the differentiation of mitral insufficiency from mitral stenosis. Reports have been published pointing out apparent discrepancies in the type of V wave found in the pulmonary artery wedge pulse contours in patients with mitral valve disease.

The detection of gross mitral insufficiency prior to operation still remains a problem of considerable importance in the absence of an operation for the relief of mitral insufficiency, comparable with that of commissurotomy for mitral stenosis. Clinical, electrocardiographic, and roentgenologic examinations are of great value in the differential diagnosis, but are not always sufficiently accurate to prevent fruitless thoracotomy and cardiotomy being carried out on some patients in whom the predominant or only lesion is mitral insufficiency.

In an effort to detect characteristic differences, particularly in reference to the characteristics of the pulmonary artery wedge pressure, an analysis was made of the hemodynamic data obtained at cardiac catheterization in 24 patients with mitral stenosis and 20 patients with mitral insufficiency. In addition, a study was made of similar data obtained in three patients with aortic stenosis in left ventricular failure and 16 healthy subjects.

SELECTION OF PATIENTS

In all of the 24 patients with mitral stenosis, the lesion was of severe degree (Grade 3 or 4) and the diagnosis was confirmed during commissurotomy carried out by Dr. J. W. Kirklin. Minimal mitral insufficiency was detected in 4 of these patients and was believed

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to be hemodynamically insignificant both before and after incision and finger fracture of the commissures. A mild degree of tricuspid stenosis was present in one additional instance (Patient D). The 24 patients with mitral stenosis were selected from the larger group of patients who had undergone operation and in whom catheterization data were available, by reason of the fact that in each of these 24 patients, a blood sample of high saturation (greater than 95 per cent) was withdrawn through the catheter wedged in a peripheral branch of the pulmonary artery. In 4 of the patients the data were obtained at cardiac catheterization subsequent to mitral commissurotomy. Fifteen of the patients had a normal sinus rhythm and the remainder had atrial fibrillation.

The diagnosis was made at operation in 9 of the 20 patients with mitral insufficiency. In each of these 9 patients the surgeon believed mitral insufficiency to be the predominant lesion, and in 7 it was felt to be the only significant lesion so that no commissurotomy was performed. In the remaining 2 patients (d and g) who had undergone operation, some degree of mitral stenosis was present in addition to the predominant insufficiency, and the commissures were incised. The data reported in these 2 patients were obtained at postoperative cardiac catheterization when the predominant lesion was mitral insufficiency.

In 3 patients (e, j, and l) the diagnosis of mitral insufficiency was made at necropsy carried out by Dr. J. E. Edwards. In 2 of these, the mitral insufficiency was felt to be congenital and was associated with other cardiovascular lesions. In the remaining patient on whom necropsy was performed (who had in addition an atrial septal defect) the mitral insufficiency was considered to be the result of previous bacterial endocarditis.

In 8 patients (m through t) the diagnosis of predominant mitral insufficiency was felt to be reasonably certain on clinical grounds and for this reason operation was refused without recourse to the data obtained at cardiac catheterization. The criteria on which the clinical diagnosis of predominant mitral insufficiency was based in these 8 patients included most or all of the following: A prolonged apical systolic murmur of at least Grade 2 intensity (on the basis of 1 to 4) on auscultation; the roentgenologic findings of left ventricular enlargement and pulsation of the left atrium synchronous with ventricular systole, in addition to left atrial enlargement; and evidence of left ventricular hypertrophy in the electrocardiogram, in the absence of detectable aortic valve disease. Nine of the patients with mitral insufficiency had a normal sinus rhythm, 10 had atrial fibrillation and 1 had a nodal rhythm.

In each of the 3 patients with left ventricular failure there was a normal sinus rhythm and the only valvular lesion present was an aortic stenosis, proved at operation in one instance, and at necropsy (without previous operation) in the other two instances.

The normal subjects were 16 healthy male physicians.

METHODS

During cardiac catheterization, the procedure and recording systems were those previously described.[6, 7] The catheters used were of the Cournand type, size No. 4 to No. 7 with three terminal orifices (bird's-eye tip without terminal grooves). Pressures were recorded via specially adapted strain-gauge manometers[7] (Model P6-15G-250, Statham Co.). The dynamic response characteristic of this type of gauge when used in conjunction with cardiac catheters has been described previously.[8]

Zero level for all pressure measurements was the midanteroposterior chest level, at the sternal end of the third intercostal space. Integrated mean pulmonary artery and mean pulmonary artery wedge pressures were measured with a compensating polar planimeter during one or two complete respiratory cycles. The average peak of the systolic (V) wave of the pulmonary artery wedge pressure pulse was obtained from measurement of each individual V-wave peak during the same respiratory cycles as were used for measurement of the mean pulmonary artery wedge pressure.

Blood samples were analyzed for oxygen content and capacity by the method of Van Slyke, and cardiac output was calculated by the Fick principle. Exercise was performed in the supine position using a bicycle ergometer.[7] The period of exercise was usually less than five minutes and varied with the patient's tolerance.

TABLE I. DATA OBTAINED

PATIENT	ADDITIONAL DIAGNOSIS AT OPERATION	AGE	SEX	SURFACE AREA, METER²	RHYTHM	HEART RATE		CARDIAC INDEX L./MIN./M.²		RIGHT ATRIAL MEAN PRESSURE, MM. HG	PULMONARY ARTERY PRESSURE, MM. HG			
											REST		EXERCISE	
						REST	EXERCISE	REST	EXERCISE		MAX. MIN.	MEAN	MAX. MIN.	MEAN
														MITRAL
A	-------	38	M	1.82	A.F.	96	—	3.2	—	11	110/50	72	—	—
B	-------	27	F	1.58	S.R.	72	168	2.3	1.8	7	108/45	84	165/88	112
C	Grade 0-1 mitral insufficiency	33	F	1.52	S.R.	96	108	3.0	3.0	5	85/45	63	90/50	67
D	Grade 1 tricuspid stenosis	40	F	1.51	A.F.	75	—	1.9	—	17	85/41	56	—	—
E	Grade 1 mitral insufficiency	38	M	1.72	S.R.	96	108	2.4	2.3	10	68/39	50	95/55	68
F	-------	23	F	1.53	S.R.	108	120	3.9	4.3	4	44/28	33	60/37	47
G	-------	37	F	1.56	S.R.	72	96	2.6	3.2	7	80/40	56	114/55	85
H	-------	25	F	1.65	S.R.	84	144	3.1	5.1	3	49/27	35	101/51	68
I	-------	33	F	1.49	S.R.	84	150	2.8	5.5	5	74/36	51	125/60	83
J	Grade 0-1 mitral insufficiency	41	M	1.68	S.R.	84	120	2.0	2.0	2	76/42	54	122/72	94
K	-------	43	M	2.00	A.F.	78	96	1.7	2.4	6	44/28	35	76/42	57
L	-------	39	F	1.53	S.R.	66	156	2.6	4.4	7	34/22	26	94/71	80
M	-------	38	F	1.50	S.R.	78	150	2.5	4.0	4	40/28	36	125/66	90
N	-------	43	M	1.73	S.R.	96	108	3.1	3.7	3	56/29	39	117/55	80
O	-------	36	M	1.79	A.F.	48	60	2.2	2.5	6	48/20	28	66/26	48
P	-------	36	F	1.53	A.F.	90	150	3.2	4.4	8	36/20	28	59/37	46
Q	-------	44	F	1.76	A.F.	72	132	2.4	3.9	6	35/22	29	72/39	54
R	-------	37	M	1.92	A.F.	60	78	2.2	3.1	8	38/25	28	56/24	39
S	-------	35	F	1.69	S.R.	84	102	2.8	3.9	5	36/16	24	44/25	35
T	-------	52	M	1.64	A.F.	84	96	1.9	2.3	5	51/23	35	74/36	48
U	-------	39	F	1.51	S.R.	66	90	2.7	4.1	5	34/20	25	87/57	72
V	-------	40	F	1.40	S.R.	72	102	2.7	3.5	3	49/26	36	100/47	68
W	-------	27	F	1.57	S.R.	84	138	3.2	4.1	4	32/15	20	63/30	44
X	Grade 0-1 mitral insufficiency	48	F	1.64	A.F.	48	114	1.5	2.9	4	26/13	18	67/32	47
Average (24)		37		1.64		79	118	2.6	3.5	6	56/29	40	90/48	65
														MITRAL
a	Grade 1 mitral stenosis	48	F	1.58	A.F.	100	—	2.5	—	15	86/39	60	—	—
b	-------	35	M	1.87	S.R.	78	100	2.8	3.9	7	70/30	50	107/56	80
c	Endocardial sclerosis, patent ductus, coarctation	5	M	—	S.R.	160	—	—	—	8	92/66	80	—	—
d†	Grade 1-2 mitral stenosis	33	F	1.62	A.F.	54	140	2.2	3.3	12	58/25	38	100/49	73
e	-------	36	F	1.70	A.F.	90	138	2.7	4.1	3	35/20	26	60/40	48
f	Grade 0-1 mitral stenosis	27	M	1.84	S.R.	84	96	3.1	3.8	4	46/24	34	79/41	58
g†	Grade 1-2 mitral stenosis	41	M	1.70	A.F.	72	94	2.7	3.0	9	60/35	45	79/34	52
h	-------	32	F	1.54	S.R.	102	—	3.1	—	7	32/19	25	—	—
i	-------	33	F	1.81	A.F.	80	126	2.4	3.1	7	33/18	24	48/26	35
j	Atrial septal defect, healed bacterial endocarditis	48	F	1.59	S.R.	110	—	2.5	—	16	127/43	78	—	—
k	-------	34	F	1.55	A.F.	72	114	1.9	2.5	9	29/14	19	47/27	35

RADIAL ARTERY PRESSURE, MM. HG		MITRAL VALVE AREA, CM.^{2}	PULMONARY ARTERY WEDGE PRESSURE, MM. HG				ECG Q-T INTERVAL, SECOND	RATIO Q-V PEAK / Q-T	RATIO Q-V ONSET / Q-T	RATIO RY/V[9]	LEFT ATRIAL MITRAL VALUE[10]
			REST		EXERCISE						
REST MAX. / MIN.	EXERCISE MAX. / MIN.		AVERAGE V PEAK	MEAN	AVERAGE V PEAK	MEAN					
STENOSIS*											
170/85	—	0.8	54	46	—	—	0.33	1.04	0.52	0.90	34.6
108/56	118/75	0.6	40	34	50	42	0.35	1.24	0.85	0.72	16.7
123/64	127/70	0.7	36	33	—	—	0.33	1.10	0.48	1.57	34.1
95/59	—	0.4	38	32	—	—	0.37	1.11	0.24	0.81	36.4
88/60	99/65	0.7	35	30	39	33	0.32	1.13	0.69	1.49	24.3
116/66	145/68	1.0	35	30	—	—	0.29	1.23	0.42	1.77	31.3
100/57	115/70	0.7	36	29	54	44	0.38	1.17	0.84	0.92	33.3
120/62	155/85	1.0	31	29	55	47	0.38	1.09	0.77	1.22	17.9
112/62	125/75	0.9	34	28	—	—	0.30	1.14	0.60	1.70	35.0
118/79	132/80	0.6	30	26	40	35	0.35	1.13	0.70	1.57	52.6
114/69	115/67	0.6	28	23	—	—	0.33	1.21	0.92	0.79	32.4
122/80	166/97	0.9	27	23	—	—	0.41	1.21	0.84	1.46	43.3
106/64	109/66	0.5	25	22	—	—	0.36	1.20	0.67	0.92	25.0
112/56	130/70	1.1	27	22	46	38	0.34	1.14	0.77	2.62	31.0
122/65	149/69	0.7	24	21	34	27	0.39	1.16	0.71	0.54	24.2
125/72	127/77	1.0	25	20	44	35	0.31	1.11	0.73	1.02	39.3
144/77	198/95	1.0	26	20	60	42	0.39	1.09	0.53	1.24	37.0
130/62	158/80	0.8	22	18	32	26	0.42	1.13	0.67	0.76	18.2
128/67	156/81	1.2	21	18	36	32	0.35	1.20	0.77	1.15	26.1
130/72	142/90	0.7	20	16	28	23	0.40	1.20	0.69	0.63	21.1
94/45	125/65	1.2	19	16	37	32	0.42	1.22	0.71	0.73	39.1
102/62	140/78	1.1	15	14	35	27	0.40	1.20	0.70	1.38	36.0
119/63	149/77	1.6	17	14	43	34	0.35	1.16	0.75	2.08	53.3
100/85	120/100	0.7	17	12	—	—	0.41	1.20	0.54	0.76	45.0
117/66	136/77	0.9	28	24	42	34	0.36 ± 0.01	1.16 ± 0.01	0.67 ± 0.03	1.20	32.8
INSUFFICIENCY‡											
210/90	—	—	55	39	—	—	0.35	0.90	0.41	1.52	50.0
108/66	120/70	—	42	28	45	30	0.33	1.23	0.70	2.47	53.1
100/58	—	—	36	27	—	—	0.30	0.99	0.85	3.78	33.3
140/64	187/87	—	37	26	47	34	0.47	1.16	0.55	0.51	42.4
127/64	133/71	—	34	24	57	42	0.38	1.16	0.35	1.90	66.7
96/54	119/65	—	32	23	—	—	0.37	1.18	0.76	2.27	43.1
120/70	134/69	—	34	22	42	30	0.34	1.20	0.63	1.68	44.1
110/60	—	—	25	19	—	—	0.34	1.12	0.66	3.02	46.4
125/73	135/87	—	24	18	—	—	0.39	0.96	0.63	1.03	37.5
101/74	—	—	22	17	—	—	0.27	1.03	0.48	1.22	30.4
138/83	162/97	—	18	15	30	23	0.37	1.20	0.76	0.85	33.3

TABLE I—

PATIENT	ADDITIONAL DIAGNOSIS AT OPERATION	AGE	SEX	SURFACE AREA, METER²	RHYTHM	HEART RATE		CARDIAC INDEX L./MIN./M.²		RIGHT ATRIAL MEAN PRESSURE, MM. HG	PULMONARY ARTERY PRESSURE, MM. HG			
											REST		EXERCISE	
						REST	EXERCISE	REST	EXERCISE		MAX. MIN.	MEAN	MAX. MIN.	MEAN
l	Patent ductus arteriosus, ventricular septal defect	34	F	—	Nodal	102	—	—	—	8	110/74	90	—	—
m	CLINICAL	28	M	1.57	S.R.	96	—	2.9	—	8	78/39	55	—	—
n	CLINICAL	23	F	1.64	S.R.	84	144	2.8	2.9	4	68/41	57	103/57	74
o	CLINICAL	22	F	1.62	A.F.	102	162	1.6	2.0	15	85/45	65	105/52	74
p	CLINICAL	45	F	1.53	A.F.	90	120	2.1	2.2	10	40/20	31	62/39	50
q	CLINICAL	32	M	1.97	A.F.	80	130	2.2	3.7	8	38/18	27	65/34	45
r	CLINICAL	44	M	1.85	A.F.	70	72	2.6	3.1	3	39/21	28	74/36	49
s	CLINICAL	11	M	1.28	S.R.	90	—	3.9	—	5	43/21	31	—	—
t	CLINICAL	20	F	1.42	S.R.	114	130	3.1	4.0	2	33/15	24	36/18	28
Average	(20)	32		1.65		92	120	2.6	3.2	8	60/31	44	74/39	54
														LEFT
1	-------	55	M	1.66	S.R.	90	—	2.0	—	15	93/42	59	—	—
2	-------	43	M	1.64	S.R.	100	—	2.2	—	4	68/40	50	—	—
3	-------	46	M	1.68	S.R.	96	—	3.5	—	12	54/30	39	—	—
Average	(3)	48		1.66		95	—	2.6	—	10	72/37	49	—	—
														HEALTHY
Average	(16)	32	M	1.93	S.R.	75	116§	3.6	5.7§	6	22/12	17	32/16§	24§

*Arranged in order of mean wedge pressure.
†Catheterization data, postoperative.
‡Arranged in 3 groups, each in order of mean wedge pressure.
§Exercise values, nine healthy subjects.

The time of onset and peak of the V wave of the pulmonary artery wedge pressure pulse were measured from the Q wave of the simultaneously recorded electrocardiogram. The ratio of these values to the duration of the Q-T interval of the electrocardiogram was calculated, in an effort to minimize variations due to differences in heart rate. A correction was made in every instance for the time lag in the catheter-galvanometer system used in the recording. The necessary correction was obtained by measuring the difference in time lag of the onset of responses to a square variation in pressure for each catheter-strain gauge system, and the response of the electrocardiograph to a square wave change in voltage.

RESULTS

The data obtained at cardiac catheterization in all patients are listed in Table I. No definite distinction between the patients with mitral stenosis and those with mitral insufficiency could be found on comparison of cardiac index, heart rate, pulmonary vascular resistances, and systemic arterial pressure either during rest or during the response to exercise.

The average mean pulmonary artery wedge pressures in the two groups of patients were not significantly different nor was there a marked difference in the average peak V-wave pressures in the patients with mitral stenosis or insufficiency, respectively (Table I). However, when the average peak V-wave pressure was plotted against the mean wedge pressure, a significant correlation was found and a distinct separation between the two groups of patients was evident (Fig. 1). This relationship apparently was not affected by the type of cardiac rhythm present.

CONT'D

RADIAL ARTERY PRESSURE, MM. HG		MITRAL VALVE AREA, CM.^{2}	PULMONARY ARTERY WEDGE PRESSURE, MM. HG				ECG Q-T INTERVAL, SECOND	RATIO Q-V PEAK / Q-T	RATIO Q-V ONSET / Q-T	RATIO RY/V[9]	LEFT ATRIAL MITRAL VALUE[10]
			REST		EXERCISE						
REST MAX. / MIN.	EXERCISE MAX. / MIN.		AVERAGE V PEAK	MEAN	AVERAGE V PEAK	MEAN					
122/80	—	—	18	14	—	—	0.33	1.22	0.63	1.03	50.0
114/63	—	—	44	31	—	—	0.34	1.25	0.54	1.42	47.6
117/70	149/82	—	42	30	—	—	0.35	1.35	0.60	2.57	20.0
155/90	180/107	—	45	28	—	—	0.28	1.30	0.65	2.20	48.0
161/94	200/114	—	32	22	52	38	0.35	1.19	0.74	1.52	50.0
115/67	141/81	—	30	22	57	36	0.30	1.51	0.92	1.20	37.0
90/53	121/61	—	29	21	50	38	0.41	1.21	0.67	0.67	38.7
159/101	—	—	28	20	—	—	0.43	1.12	0.46	1.76	23.6
124/70	148/80	—	26	17	32	21	0.31	1.28	0.73	4.24	51.7
127/72	148/82	—	33	23	46	32	0.35 ± 0.01	1.18 ± 0.03	0.64 ± 0.03	1.84	42.3
VENTRICULAR FAILURE											
123/82	—	—	42	36	—	—	0.46	1.12	0.56	1.64	33.3
96/55	—	—	40	34	—	—	0.35	1.09	0.68	2.61	40.9
104/60	—	—	41	34	—	—	0.31	1.41	0.84	1.92	37.2
108/66	—	—	41	35	—	—	0.37	1.21	0.69	2.06	37.1
MALE SUBJECTS											
139/74	168/83§	—	12.5	10	—	—	0.36 ± 0.01	1.31 ± 0.03	0.83 ± 0.04	—	—

It became apparent that at equivalent elevations of mean pulmonary artery wedge pressure the peak V-wave pressure was significantly greater in mitral insufficiency than in mitral stenosis, and that when the mean left atrial pressure exceeded 20 mm. Hg there was no overlap between the two groups. Extreme examples of this characteristic difference in V-wave pressure at equivalent mean pressure levels are illustrated in Fig. 2, by comparison of the peak V-wave pressures in a patient with mitral stenosis and in another with mitral insufficiency, in each of whom the mean pulmonary artery wedge pressure was 28 mm. Hg, heart rate 90 per minute and with a normal sinus rhythm.

The disproportionate increase in peak V-wave pressure in mitral insufficiency can be demonstrated by calculating the ratio of the V-wave pressure to mean pulmonary artery wedge pressure (Fig. 3). When the mean pulmonary artery wedge pressure exceeded 20 mm. Hg this ratio was uniformly greater than 1.3 in patients with mitral insufficiency and less than 1.3 in patients with mitral stenosis. Below a mean pulmonary artery wedge pressure of 20 mm. Hg there was a considerable overlap between the groups of patients with mitral stenosis and insufficiency, and also with the group of normal subjects. In the three patients with left ventricular failure consequent to aortic stenosis, the data fell within the group typical of mitral stenosis.

In an effort to increase the ability to distinguish the two groups with left atrial mean pressures less than 20 mm. Hg, the effect of exercise on the relative magnitudes of the V wave and mean pulmonary artery wedge pressure (Fig. 4), as well as on other physiologic variables (Table II), was observed. Elevation of mean left atrial pressure by this maneuver was of some value, since at mean

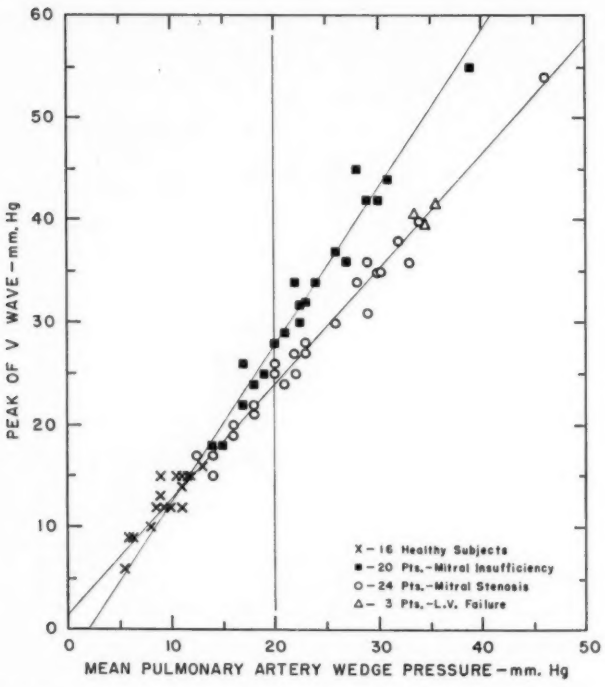
PEAK OF V WAVE – mm. Hg
MEAN PULMONARY ARTERY WEDGE PRESSURE – mm. Hg
X – 16 Healthy Subjects
■ – 20 Pts. – Mitral Insufficiency
○ – 24 Pts. – Mitral Stenosis
△ – 3 Pts. – L.V. Failure

Fig. 1.—Relationship between average peak V-wave pressure and pulmonary artery wedge mean pressure in 24 patients with mitral stenosis, 20 with mitral insufficiency, 3 with left ventricular failure, and 16 healthy subjects. A significant positive correlation between these pressures was obtained in the groups with mitral stenosis ($r = 0.99$) and mitral insufficiency ($r = 0.98$). The calculated regression equation for the group with mitral stenosis was $Y = 1.1\ X + 1.4$, and that for the group with mitral insufficiency was $Y = 1.5\ X - 2.9$ (Y = average peak of V wave and X = mean pulmonary artery wedge pressure, mm. Hg). Note that when pulmonary artery wedge mean pressures exceeded 20 mm. Hg there was no overlap between the patients with mitral stenosis and those with mitral insufficiency and that the patients with left ventricular failure fell in the mitral stenosis group.

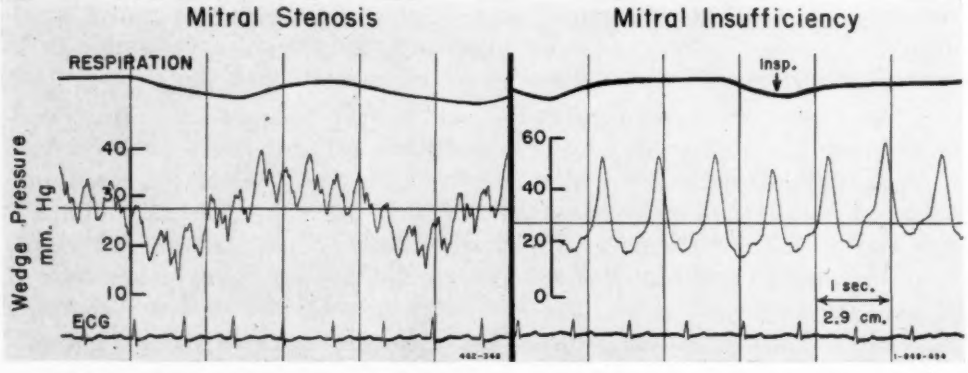

Fig. 2.—Comparison of pulmonary artery wedge pressure pulse contours in (1) a patient with mitral stenosis and (2) a patient with mitral insufficiency (each diagnosis confirmed at operation). Note that in each patient the integrated mean pulmonary artery wedge pressure was 28 mm. Hg and average heart rate 90 per minute with normal sinus rhythm. In the patient with mitral stenosis the average peak V wave measured 34 mm. Hg and in the patient with mitral insufficiency it measured 50 mm. Hg, in these recordings.

pulmonary artery wedge pressure levels above 25 mm. Hg, a clear-cut distinction between the two groups again was observed (Fig. 4), with the exception of one postoperative instance of mitral stenosis (Patient Q) in which the anticipated significant degree of relief was not obtained by mitral commissurotomy.

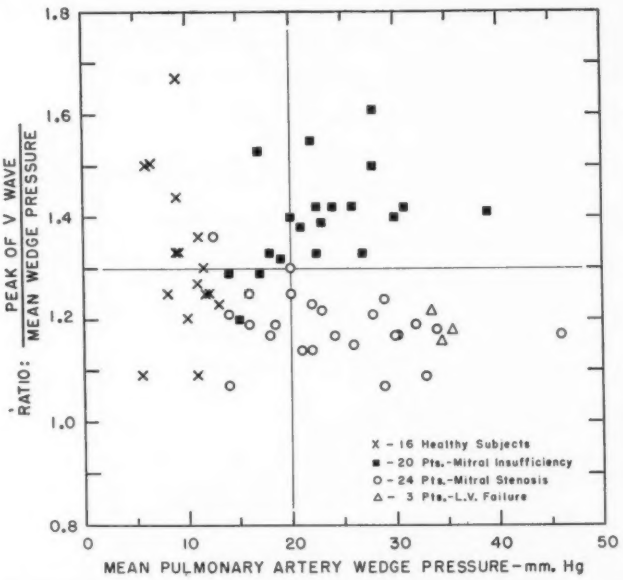

Fig. 3.—Relationship between pulmonary artery wedge mean pressure, and the ratio $\frac{\text{average peak V-wave pressure}}{\text{mean wedge pressure}}$ in 24 patients with mitral stenosis, 20 patients with mitral insufficiency, 3 patients with left ventricular failure, and 16 healthy subjects. Note that when pulmonary artery wedge mean pressures exceeded 20 mm. Hg the ratio uniformly exceeded 1.3 in the patients with mitral insufficiency, and was uniformly less than 1.3 in the patients with mitral stenosis. The patients with left ventricular failure fell in the mitral stenosis group.

TABLE II. EFFECT OF EXERCISE ON PHYSIOLOGIC VARIABLES IN PERSONS WITH MITRAL STENOSIS AND IN PERSONS WITH MITRAL INSUFFICIENCY

	MITRAL STENOSIS (15)		MITRAL INSUFFICIENCY (9)	
	REST	CHANGE WITH EXERCISE (MEAN ± S.E.$_m$)	REST	CHANGE WITH EXERCISE (MEAN ± S.E.$_m$)
Heart rate (beats/minute)	78	+ 35 ± 6	80	+ 35 ± 7
Cardiac index (liters/min./M.^{2})	2.6	+ 0.7 ± 0.2	2.5	+ 0.8 ± 0.2
Stroke index (ml./beat/M.^{2})	34	− 3 ± 2	32	− 2 ± 2
Radial artery pressure (mm. Hg)	116/64	+ 23/13 ± 4/3	125/70	+ 26/10 ± 5/3
Mean pulmonary artery pressure (mm. Hg)	38	+ 26 ± 3	32	+ 19 ± 3
Mean pulmonary artery wedge pressure (mm. Hg)	22	+ 12.5 ± 1.5	22	+ 10.5 ± 2
Average peak V-wave pressure (mm. Hg)	26	+ 16 ± 2	31.5	+ 14.5 ± 3
Pulmonary arteriolar resistance (dynes sec. cm.$^{-5}$)	350	+200 ± 100	200	+100 ± 50
Total pulmonary resistance (dynes sec. cm.$^{-5}$)	750	+300 ± 100	600	+150 ± 50

The patient died as a result of arterial embolism three months after commissurotomy. At necropsy a large thrombus was found in the left atrium, originating in the atrial wall at the site of amputation of the atrial appendage. There was no mitral insufficiency. It is considered possible that an alteration in pressure-volume characteristics of the left atrium was produced by the thrombus, as suggested by the equivocal data obtained at rest (Figs. 1 and 3) and the inconsistent measurements obtained during exercise (Fig. 4) in this patient with mitral stenosis.

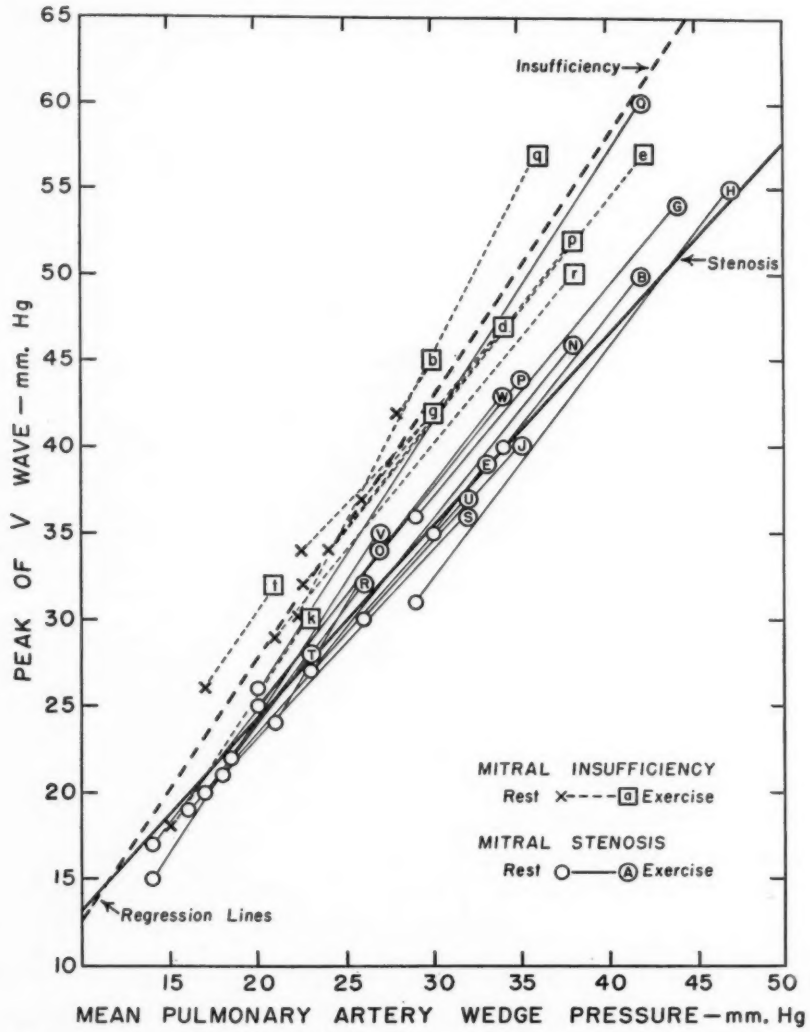

Fig. 4.—Relationship between the average peak V-wave pressure and mean pulmonary artery wedge pressure at rest and during exercise in 15 patients with mitral stenosis and 9 patients with mitral insufficiency. Note (1) that the correlation between the V-wave pressure and mean wedge pressure persisted during exercise and that, with one exception (patient Q, see text for details) the separation between the patients with mitral stenosis and the patients with insufficiency was maintained, and (2) that the light lines representing the changing values from rest to exercise in individual patients tended to parallel the regression lines (heavy solid and dashed) drawn for the resting values in each group.

An opportunity to study an effect opposite to that produced by exercise, namely, the change in left atrial pulse contour resulting from a fall in left atrial pressure, was obtained during thoracotomy and cardiotomy on Patient a with mitral insufficiency (Fig. 5). In this patient, a severe fall in systemic arterial pressure occurred during operation, almost certainly accompanied by a considerable decrease in cardiac output. There occurred very gross diminution in the level of mean left atrial pressure and the magnitude of the V wave as compared with the preoperative findings.

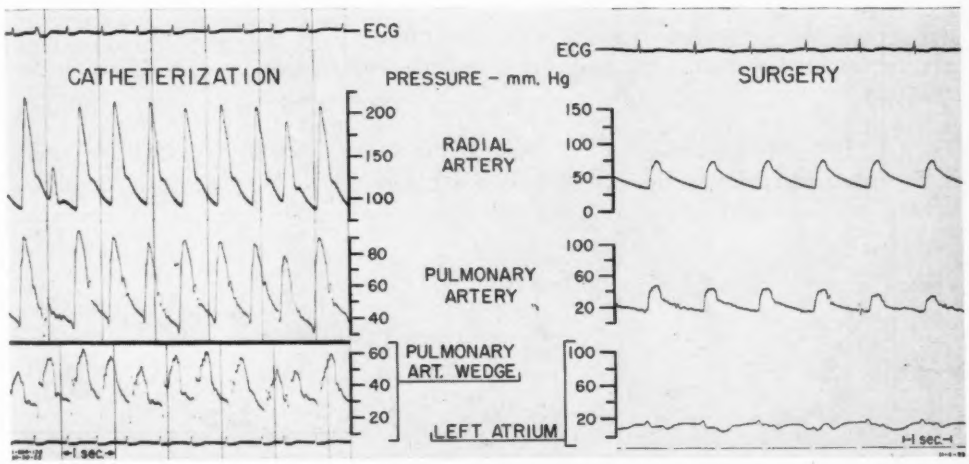

Fig. 5.—Comparison of the pulmonary artery wedge and left atrial pressure pulse contours in a patient (a) with mitral insufficiency and atrial fibrillation studied during preoperative cardiac catheterization (left panel) and during operation (right panel) respectively. Note that during preoperative catheterization, when the systemic blood pressure averaged 210/90 mm. Hg, the pulmonary artery wedge mean pressure averaged 39 mm. with a disproportionately large V-wave peak, averaging 55 mm., and that during operation, when the systemic blood pressure fell to 75/45 mm. and the mean left atrial pressure averaged 12 mm., the amplitude of the V wave was markedly diminished and was not suggestive of the presence of mitral insufficiency.

In view of recent publications suggesting methods designed to distinguish mitral stenosis and insufficiency,[9, 10] the methods described in these reports were applied to the pulmonary artery wedge pressure pulse contours of the patients with mitral stenosis and mitral insufficiency studied in the present series. Measurements were made as far as possible at the end of expiration, when there was no respiratory fluctuation in pressures, although patients were not made to hold the breath in the expiratory position for any length of time. Also, zero reference point for pressure measurements was midchest level at the anterior end of the third intercostal space, while that point, in the studies of both Owen and Wood,[9] and of Allison and Linden,[10] was at the level of the manubrium sterni. This produces a relative increase in the denominator of our values for their formulas. An accurate reproducibility of values from pulse to pulse was not achieved consistently with either method, particularly in patients with arrhythmia. The late diastolic dip in pressure described by Owen and Wood was not found consistently and in many patients was indistinguishable by reason of motion artifact in the pressure recordings. The data obtained on comparison of the factors Ry and "left atrial mitral value" with

the pulmonary artery wedge mean pressure are presented in Fig. 6. Of the methods described by Owen and Wood, and by Allison and Linden, neither provided an adequate distinction between the patients with mitral stenosis and mitral insufficiency in this series. It is appreciated that the method of Owen and Wood is designed to measure the degree of obstruction at the mitral valve by a measurement of the rate of "Y" descent of the V wave (Fig. 7). The values for Ry/V (Table I) less than 1.6 are consistent with the findings of Owen and Wood in 20 of the 24 patients with mitral stenosis, as also are 10 of the 20 values for Ry/V greater than 1.6 in the patients with mitral insufficiency. Values greater than 1.6 were also obtained in the three patients with left ventricular failure in whom there was no obstruction at the mitral valve (Table I).

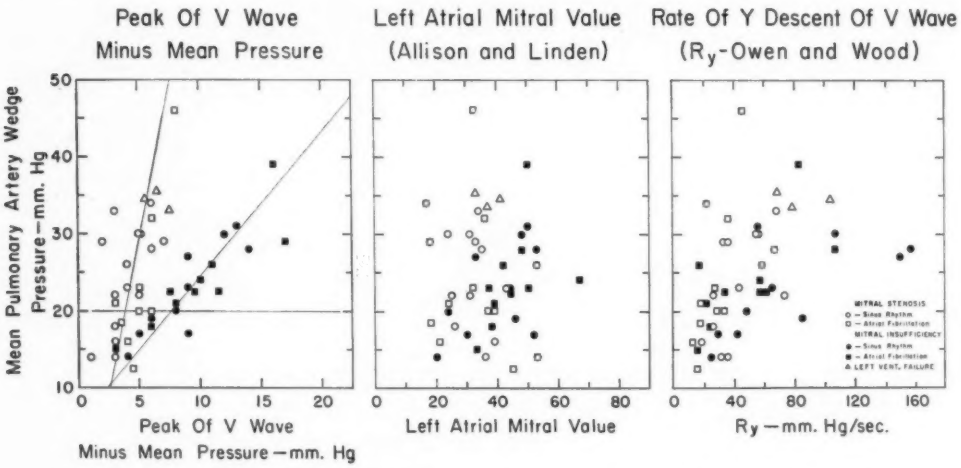

Fig. 6.—Comparison of three different methods employed for differentiation of mitral insufficiency from stenosis on the basis of pulmonary artery wedge or left atrial pulse contours. Note that only the left hand panel shows a clear-cut separation of the patients with mitral stenosis from those with mitral insufficiency, which was uniform when the mean wedge pressure exceeded 20 mm. Hg.

Other authors[11, 12] have described differences in the contour and duration of the pulmonary artery wedge pulse in patients with mitral insufficiency compared with mitral stenosis (for example, the "tentlike" waves of Biörck and associates[11]). In order to obtain data in this regard, the times of onset and peak of the V wave of the pulmonary artery wedge pressure pulse after onset of Q wave of the electrocardiogram were measured in all patients with mitral stenosis and insufficiency. In an attempt to minimize variations due to differences in heart rate between individual patients, these times were related to the duration of the Q-T intervals of the electrocardiogram recorded simultaneously with the pulmonary artery wedge pressure pulses, and the ratios $\frac{\text{Q-V peak}}{\text{Q-T}}$ and $\frac{\text{Q-V onset}}{\text{Q-T}}$ were calculated (Table I). No measurement was made of the time of diastolic decline of the V-wave pressure since this varied grossly with the heart rate, particularly in patients with auricular fibrillation, in whom there was a marked difference from pulse to pulse.

In both mitral stenosis and mitral insufficiency the peak of the V wave occurred at a relatively fixed time with regard to the Q-T interval (mitral stenosis 1.16 ± 0.01, mitral insufficiency 1.18 ± 0.03). This relatively uniform value is an aid in the identification of the V wave of the pulmonary artery wedge pressure pulse contour. A significant difference between the relative times of onset of the V waves was not observed between the patients with mitral stenosis and those with mitral insufficiency (mean $\frac{\text{Q-V onset}}{\text{Q-T}}$ for mitral stenosis, 0.67 ± 0.03, mitral insufficiency 0.64 ± 0.03). It was not always possible to decipher a definite C wave in patients with mitral stenosis and patients

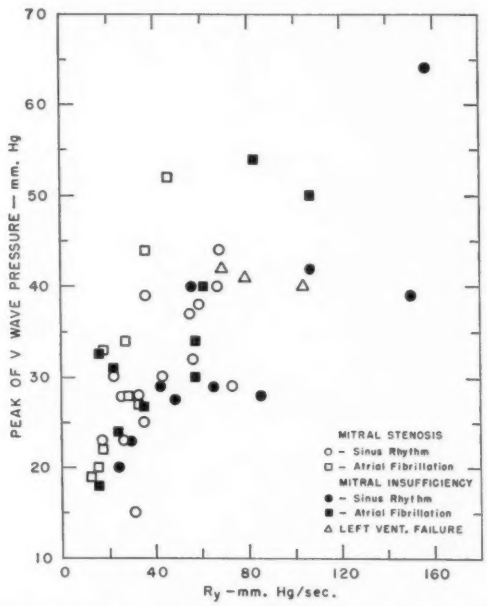

Fig. 7.—Comparison of peak V-wave pressure and rate of Y descent of the V wave of individual pulses in the manner suggested by Owen and Wood.[9] The differentiation of individual patients with stenosis or insufficiency is far from uniform.

with mitral insufficiency, since this wave is usually of considerably lesser magnitude than the V wave and may be superimposed on the onset of the V wave in some cases of mitral insufficiency. A small C wave often may be obscured by the artifact usually seen in pressures recorded via a catheter threaded through the beating heart. On the contrary, the V wave was very rarely not distinct, particularly in patients with elevated left atrial pressures. In all patients except one in the present series (Patient 1) the V wave was easily decipherable.

A comparative study of pulmonary artery wedge pressure pulse contours in patients with mitral stenosis and mitral insufficiency revealed findings which have resembled those previously described.[13] Most patients with mitral insufficiency showed an absence of the usual decline in pressure between the C and

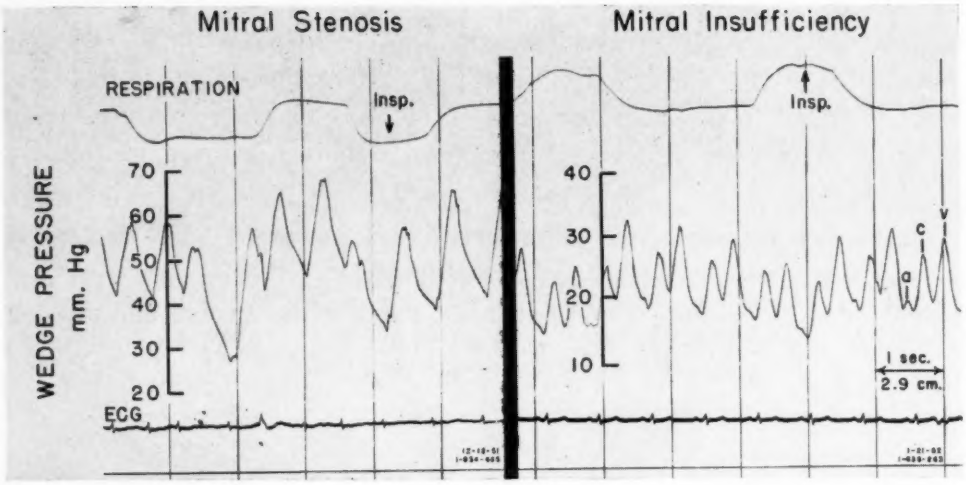

Fig. 8.—Comparison of pulmonary artery wedge pressure pulse contours in a patient with mitral stenosis (Patient A, left panel) and a patient with mitral insufficiency (Patient F, right panel) (diagnosis in each case confirmed at operation). Note that, contrary to what has been described as being typical of pulmonary artery wedge or left atrial pulse contours in patients with mitral stenosis and mitral insufficiency, in the patient with mitral stenosis (who also had atrial fibrillation) no clearly defined C wave is distinguishable, whereas in the patient with mitral insufficiency a very clearly defined S wave is distinguishable, whereas in the patient with wedge pressure pulse.

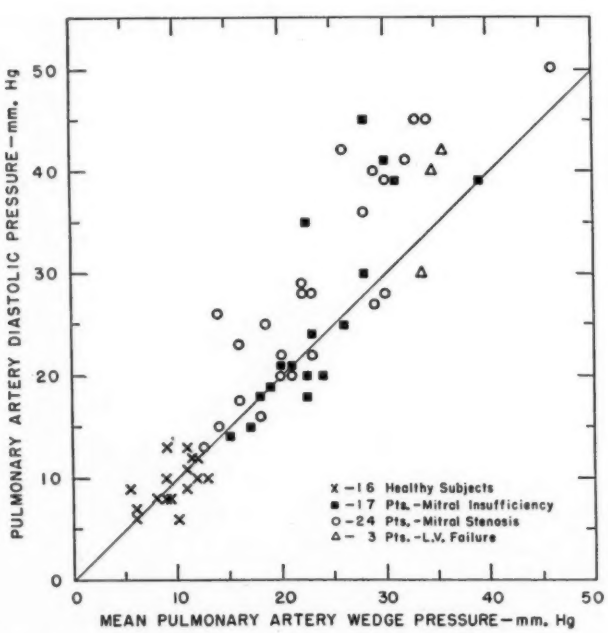

Fig. 9.—Relationship between pulmonary artery diastolic pressure and pulmonary artery wedge mean pressure in 24 patients with mitral stenosis, 17 patients with mitral insufficiency, 3 patients with left ventricular failure, and 16 healthy subjects. Note that in the healthy subjects these pressures were similar, as they were in patients with mitral stenosis and insufficiency with pulmonary artery wedge mean pressures below 20 mm. Hg. The higher mean wedge pressures, however, were associated with disproportionately high pulmonary artery diastolic pressures. (Patients c, j, and l, with intracardiac shunts, not included.) (Further discussion in text.)

V waves of the wedge pulse contour, whereas such a decline was usually evident in cases of mitral stenosis when the C wave was not obscured by artifact (Fig. 2). However, deviations from the expected type of contour were found in each group (Fig. 8).

It also was considered of interest to determine the relationship between the diastolic pressure in the pulmonary artery and the mean pulmonary artery wedge pressure in all the present series of subjects (Fig. 9). At normal or slightly elevated levels of mean pulmonary artery wedge pressure, all points were found to lie close to the line of identity. With elevated left atrial pressures a greater proportionate increase in the diastolic level of the pulmonary artery pressure was evident. The divergence from the line of identity between pulmonary artery diastolic and pulmonary mean wedge pressures appeared to begin about the 20 mm. Hg level.

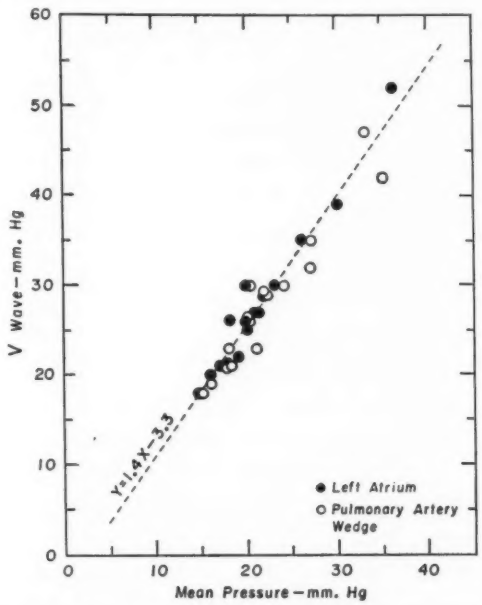

Fig. 10.—Comparison of relationship between V-wave pressure and mean pressure in simultaneous recordings of left atrial and pulmonary artery wedge pressures during open thoracotomy, in 16 patients undergoing surgical alleviation of mitral stenosis. Note that the relationship between peak V-wave pressure and mean pressure for left atrial and pulmonary artery wedge pressure pulses is not significantly different. However, the average calculated regression equation for these data ($Y = 1.4\ X - 3.3$) has a statistically significant steeper slope than that derived from the pulmonary artery wedge pulses obtained at routine cardiac catheterization in the group of 24 patients with mitral stenosis (Fig. 1).

Finally, as a means of comparing the value of pulmonary artery wedge and directly recorded left atrial pulses in differentiating mitral stenosis and mitral insufficiency, relationships between average peak V-wave pressures and mean pressures in the left atrium and in the pulmonary artery wedge position were determined in a group of 16 patients with mitral stenosis in whom simultaneous recordings of pulmonary artery wedge and left atrial pulse contours were obtained at the time of mitral commissurotomy (Fig. 10).[1]

There was no statistically significant difference between the regression lines based on these simultaneously recorded pulses. However, the slopes of these regression lines calculated from pressures recorded during open thoracotomy differed significantly from that derived from the values obtained at routine cardiac catheterization in the group of 24 patients with mitral stenosis (Fig. 1).

COMMENT

On comparison of the hemodynamics of patients with mitral stenosis and of those with mitral insufficiency, the only distinguishing feature which has been found to be of sufficient uniformity to be of practical diagnostic value is the disproportionate increase in average peak V-wave pressure, at equivalent elevations of left atrial pressure, in the patients with mitral insufficiency.

The differentiation of mitral stenosis and mitral insufficiency by this method rests on the validity of the pulmonary artery wedge pressure pulse contour as a representation of the left atrial pressure pulse. It would be expected that recordings of pulses directly from the left atrium by needle puncture or introduction of a catheter[1, 2, 10, 14, 15] would give more exact representations of the true left atrial pulse contour than would recordings of pulmonary artery wedge pressure pulses. Nevertheless the pulmonary artery wedge pulse has been demonstrated to be a reasonably accurate reproduction of the left atrial pulse, both in pressure and in contour, when these pressures were recorded simultaneously,[1] and the closest similarity was obtained when left atrial pressures were elevated, as in mitral valve disease.

The criteria used for deciding that a catheter tip was in fact wedged in a peripheral branch of a pulmonary artery have been previously described: (1) The recorded pressure pulse resembles a wedge pressure contour. (2) During fluoroscopy the catheter tip usually is seen to be situated in the peripheral lung field. (3) When the catheter manometer system is subjected to a transient stepwise increase in pressure by flushing with saline solution, the pressure returns immediately to the previous wedge pressure contour. (4) Wedge pressure pulses recorded from different positions in the same or opposite lung are closely similar. (5) When the catheter is withdrawn from the wedge position, typically, after the slack in the catheter shaft is taken up, the tip is seen to snap free suddenly, simultaneously with the sudden increase to pulmonary artery pressure. (6) It is preferable that blood samples withdrawn with the tip of the catheter in the wedge position be fully saturated with oxygen. In regard to this last criterion it has been found that frequently no blood sample could be obtained; occasionally that the blood sample was desaturated; or, rarely, that it was identical in oxygen saturation to mixed venous blood. Later, or previously in many such patients, identical pressures have been recorded, and fully saturated samples have been obtained when the tip of the catheter was wedged in different locations in the same, or opposite, lung. Therefore it is not essential to have a sample of blood fully saturated with oxygen before a given tracing can be accepted as representing pulmonary artery wedge pressure.[16]

It has been the objective in every case to obtain several recordings of the pulmonary artery wedge pulse from different sites in the same or opposite lung, together with blood samples. It has been found that pressures, contours, and saturation of blood samples are not always completely reproducible.[17] When variations have been obtained in pressure or contour, a pulmonary artery wedge pressure tracing was selected which conformed most closely to the majority of the recordings, or preferably from a position which yielded a fully saturated blood sample.

The finding of a disproportionate increase in average peak V-wave pressure in the patients with mitral insufficiency appears logical on consideration of the pressure-volume characteristics of the left atrium.[18, 19] When left atrial pressure is elevated, the addition of a regurgitant volume of blood during ventricular systole should cause the production of a relatively larger systolic (V) wave than when left atrial pressure is not elevated, or when there is no regurgitant volume of blood into the left atrium even though left atrial pressure is elevated as in mitral stenosis and left ventricular failure due to aortic valve disease. This reasoning is borne out by the measurements reported herein.

It has not been possible by clinical examinations alone to predict accurately, in our experience, the relative degrees of stenosis and insufficiency when both are present. For this reason, the data on patients with mitral insufficiency in whom the diagnosis was made by clinical examination alone have not been subdivided into groups as Owen and Wood[9] have done; instead there has been included only the group considered by clinical examinations to have predominant mitral insufficiency.

Since the data in the present study were obtained from patients in whom mitral insufficiency or mitral stenosis was the predominant lesion, it is apparent (Fig. 1) that a considerable possibility of overlap might occur in patients having coexistent mitral stenosis and insufficiency of significant degree. The coexistence of mitral stenosis and insufficiency, each of significant degree, is a less common finding than the presence of a relatively "pure" lesion, a stenosis with a minor degree of insufficiency, or a severe insufficiency without significant stenosis. It is anticipated that such lesions will be diagnosed possibly more often in the future, since it is the present custom to submit to cardiac catheterization only those patients with mitral valve disease in whom there is some doubt regarding mitral stenosis being the only lesion. The relationship between mean-wedge and V-wave pressure has been employed recently in this laboratory to aid in the prediction of the major lesion in patients suspected of having coexistent mitral stenosis and insufficiency, and who are being considered as candidates for mitral commissurotomy. The findings at operation in these patients have followed the pattern suggested by the present data, and the points representing such patients were usually intermediate in position between the groups representing stenosis and insufficiency (Fig. 1).

Ideally it would be of considerable value to be able to distinguish the hemodynamic features of mitral stenosis and insufficiency with resting levels of mean left atrial pressure less than 20 mm. Hg. The presented data suggest

that no such accurate distinction is possible, and indeed that the distinction between mitral stenosis and insufficiency is made by cardiac catheterization when it is usually least difficult clinically. However, even with elevated left atrial mean pressure, the diagnosis of mitral insufficiency on clinical examinations alone is not always certain and laboratory assistance is welcomed. In addition, with mean left atrial pressures less than 20 mm. Hg, the patients are less likely to have severe symptoms and thus are less urgent candidates for consideration of surgical treatment, so that it is at the higher levels of mean left atrial pressure that the distinction is most important. However, the results demonstrate that in patients with mean left atrial pressures less than 20 mm. Hg, some differentiation between stenosis and insufficiency can be brought out if adequate elevation of left atrial pressure can be produced through exercise (Fig. 4).

The changes in mean left atrial pressure and relative magnitude of the V wave, in response to varying levels of systemic arterial pressure in a patient (Patient a) with mitral insufficiency (Fig. 5), are consistent with those previously produced by Wiggers and Feil[20] in experimental animals with mitral insufficiency, in response to changes in systemic arterial pressure.

The method of distinguishing mitral insufficiency from mitral stenosis, as described in this paper, also has been found useful when applied to left atrial pulses recorded by left-heart catheterization using the method of Björk and Malmström[14] as modified by Fisher.[15] Measurements from left atrial pressure pulses obtained by this technique suggest that the regression equation relating peak V wave and mean pressure in these pulses will have a significantly steeper slope than that derived from pulmonary artery wedge pressure pulses in the same patients. It is of interest that the regression line obtained is closely similar to that shown in Fig. 10, also based on direct left atrial pressures, recorded, however, during open thoracotomy. The difference between this relationship for pulmonary artery wedge pressures recorded in the closed chest and for left atrial pressure pulses probably is due to damping of the peak V-wave pressure in pulmonary artery wedge pressure pulse contours so that a given level of mean left atrial pressure is associated with a lower peak V-wave pressure than that found in direct recordings of left atrial pressure.[21]

In one patient to date, a misleading deduction was made from analysis of the pulmonary artery wedge pulse as described. The measurements in this instance suggested that mitral insufficiency was the predominant lesion. However, the patient was referred for mitral commissurotomy in view of the finding at left heart catheterization of a considerable pressure gradient across the mitral valve. At operation the patient was found to have a severe degree of mitral stenosis without insufficiency.

The finding of greater proportionate increases in pulmonary artery diastolic pressures, with increases in mean pulmonary artery wedge pressures exceeding 20 mm. Hg, is consistent with the histologic "protective" changes in the pulmonary vasculature and consequent increased pulmonary "arteriolar" resistance found in patients with mitral-valve disease.[22, 23]

It is probable that at levels of left atrial pressure in excess of 20 mm. Hg the increase in left atrial pressure produced by a given volume of regurgitated blood becomes increasingly evident because of several factors: (1) Dissipation of regurgitated blood back into the total pulmonary vascular bed is impeded by: (a) the high degree of pulmonary vascular resistance which has developed, (b) the high pressure present in the arterial as well as capillary and venous vascular bed; (2) There is reduced compliance of the left atrial-pulmonary vein system at high levels of left atrial pressure.

SUMMARY

Hemodynamic data obtained at cardiac catheterization during rest and exercise have been studied in 24 patients with mitral stenosis, 20 patients with mitral insufficiency, 3 patients with left ventricular failure in the absence of mitral valve disease, and 16 healthy subjects.

The best laboratory differentiation between patients with mitral stenosis and patients with mitral insufficiency was obtained in the respective significantly different regressions between the average peak V-wave pressure and the mean pulmonary artery wedge pressure obtained in the two series of patients.

When the pulmonary artery wedge mean pressure exceeded 20 mm. Hg, the peak V-wave pressure was significantly greater in mitral insufficiency than in mitral stenosis at equivalent levels of pulmonary artery wedge pressure, and there was no overlap between the two groups. This differentiation is felt to be of diagnostic significance. When pulmonary artery wedge mean pressures were less than 20 mm. Hg, no such distinction was found between patients with mitral stenosis, patients with mitral insufficiency, and healthy subjects.

The methods described by Owen and Wood[9] and by Allison and Linden[10] to distinguish between patients with mitral insufficiency and patients with mitral stenosis failed to provide a uniform differentiation of the patients in these two groups in the present series.

The time interval from the Q wave of the electrocardiogram to the peak of the V wave of the wedge pressure pulse occupies a relatively constant position in relation to the duration of the Q-T interval in patients with mitral stenosis or insufficiency and a constant though slightly longer period in normal subjects.

No systematically significant difference was found between the time of onset of the V wave in relation to the Q-T intervals in the groups of patients with stenosis and insufficiency. The time of onset was relatively later in normal ubjects.

Pulmonary artery diastolic pressures were not systematically different from, and then exceeded, pulmonary artery wedge mean pressures when the wedge mean pressures were below and above 20 mm. Hg, respectively.

We acknowledge with gratitude the cooperation of Dr. J. W. Kirklin, who provided us with the data obtained at operation. We also acknowledge the helpful comments and criticism of Dr. H. B. Burchell, the data obtained at necropsy, provided by Dr. J. E. Edwards, and the technical assistance of Mrs. Jean Frank. The resting and exercise values for pulmonary artery and radial artery pressure in healthy subjects (Table I) are reproduced by courtesy of Dr. B. Barratt-Boyes.

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THE APPLICATION OF DIRECT BODY BALLISTOCARDIOGRAPHY TO FORCE BALLISTOCARDIOGRAPHY

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INTRODUCTION

FORCE ballistocardiography refers to the study of the recorded motion of the body relative to the *acceleration* of the mass of the blood and the circulatory viscera during the cardiac cycle. The term was introduced by Starr and co-workers[1] after experimentally determining that the ballistocardiograms of cadavers resulting from simulated systole, when recorded as displacement from a high-frequency table, were related more closely to the acceleration of the ejected fluid than to its mass, and as such appeared to be an index of cardiac force. More recently, the term "force ballistocardiography" has been adopted by Talbot and co-workers[2] and von Wittern[3] to apply to the study of ballistocardiograms recorded from a very low-frequency or aperiodic system when the motion is sensed by an accelerometer.

Burger and co-workers[4] have recently pointed out that according to certain fundamental mechanical considerations, when the body is loosely bound to its environment (low natural frequency), the deviation as recorded by an optical system or other displacement sensing devices will be proportional to the displacement of the mass of blood and viscera from its center of gravity. Also when the displacement curve is twice differentiated, either mathematically or by recording the motion by an accelerometer, the resultant record will be proportional to the force of the internal blood and visceral motions. It also was stated by Burger that when the body is tightly bound to its surroundings (high-frequency), the recorded deviation or actual displacement of the body will tend to be proportional to the acceleration of the internal circulatory mass (force). The preciseness of such relationships will be dependent upon the natural or resonant frequency of the ballistic system (including the body) and the frequency of the force that is applied to it. The damping factor is also of considerable significance in the determination of the frequency response of both amplitude and phase relationships of a given ballistic system. From these basic considerations, it is apparent that the displacement curves from an ideal high-frequency ballistic system should be identical to

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the acceleration traces from an ideal low-frequency or aperiodic ballistic system. However, there are marked differences between the displacement ballistocardiograms from the high-frequency beds and direct body systems currently employed, and the acceleration ballistocardiograms from aperiodic or very low-frequency systems. These differences have recently been reviewed and reported by Deuchar and co-workers.[5] Significant differences, both in contour of the ballistic complex or in relative wave amplitude, and also in the time relationship (phase angle) between the two systems, were observed. These variations were related to the different frequency responses of the high-frequency and the aperiodic ballistic systems. Previous reports from this laboratory have described a method of modifying the Dock technique of recording the ballistocardiogram directly from the body as it lies on a rigid surface.[6, 7] These modifications were designed to raise the natural body frequency and to increase the degree of damping, and consisted of placing the subject in a bed of putty, and applying sandbags to the shoulders and feet. The differences between records obtained by this and the original Dock technique have been described previously.[7] A comparison of the acceleration

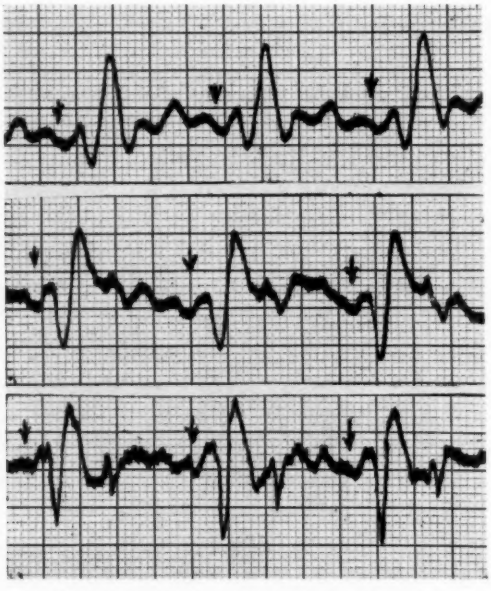


Fig. 1.—Presents the different ballistocardiographic patterns from one normal individual when taken with three types of techniques. Trace *A* represents an undamped displacement ballistocardiogram taken on a hard surface such as the floor (Dock technique). The arrows indicate the onset of the QRS complex of the electrocardiogram. Note that the tracing is somewhat oscillatory in that the movements after the J peak lack high-frequency components, and have the appearance of body oscillation. Trace *B* is the displacement record from the same patient when lying on a putty surface with sandbags placed against both feet and shoulders. Note that the oscillatory type pattern is no longer present and the waves are much sharper and there are more high-frequency phenomena present. Again, the arrows in the trace indicate the onset of the QRS complex of the electrocardiogram. Trace *C* is the record taken from the same patient on a low-frequency system (horizontal pendulum bed). The motion in this instance is recorded with an accelerometer. Note that tracings *B* and *C* both have the same general appearance and, in addition, most of the movements in the two traces occur at the same time interval in relation to the QRS complex. Note that by recording on a system with a much higher frequency response, the waves become much sharper and more distinct, and notches now appear in the traces which are not evident in the other trace. It is apparent from the tracings that increased natural body resonance as the result of applying sandbags to the feet and shoulders as in tracing *B* and recording with displacement, a tracing (at least in normal individuals) can be obtained which resembles those obtained from the low-frequency or aperiodic type suspensions.

ballistocardiograms of normal subjects from the low-frequency bed, with displacement ballistocardiograms of normal subjects by the putty bed technique, reveals marked similarity in form and time relationships. Fig. 1 illustrates an average normal Dock displacement ballistocardiogram, acceleration traces from a low-frequency bed, and the displacement trace from the putty bed technique. The differences between the records from the Dock technique and the other two traces are readily apparent. The similarity in form between the latter records is also apparent. Figs. 2 and 3 illustrate the similarity of a previously published group of acceleration ballistocardiograms from a low-frequency bed to different normal subjects as recorded from the putty bed by

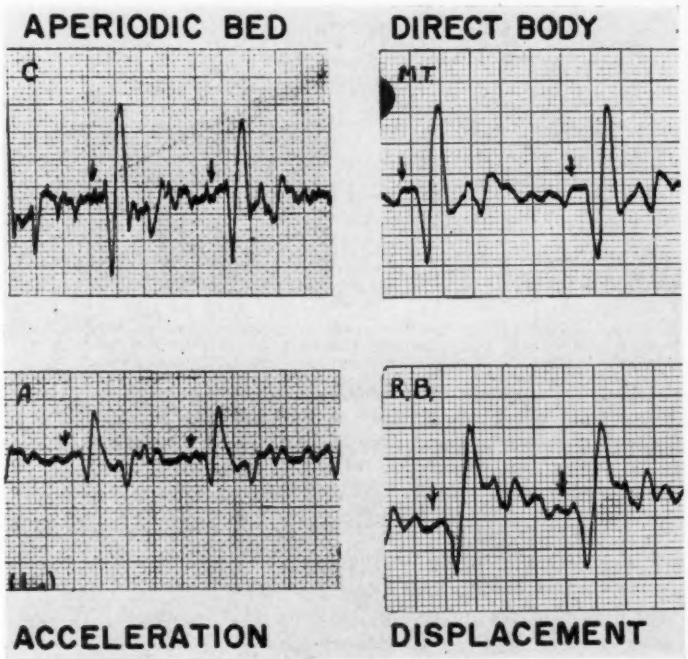


Fig. 2.—Similarities between the force ballistocardiogram taken from an aperiodic system recording the function of motion with an accelerometer as compared with the direct damped body ballistocardiogram taken with displacement technique. The traces on the left are taken from a figure previously published by Talbot and co-workers[2] (through the courtesy of the Bull. Johns Hopkins Hosp.). The arrows have been added to note the onset of the QRS complex. The traces on the left were made from a platform floating on mercury. The direct body displacement traces on the right were made with a different subject lying on a surface of putty with sandbags applied against the feet and shoulders in order to raise the natural body frequency. Note the similar configurations of both traces.

displacement. The time relations of the various waves of ballistocardiograms to the electrocardiogram can be seen to be approximately the same. It is believed that an understanding of the similarity between the displacement traces from a high-frequency, damped technique and acceleration traces from a very low-frequency, essentially undamped technique, will serve to clarify further the confusion that still exists as to the meaning of the ballistocardiograms from the various systems. It is also believed that such clarification will aid in understanding the relationship between the cardiovascular motion and the velocity and acceleration ballistocardiograms from the direct body techniques.

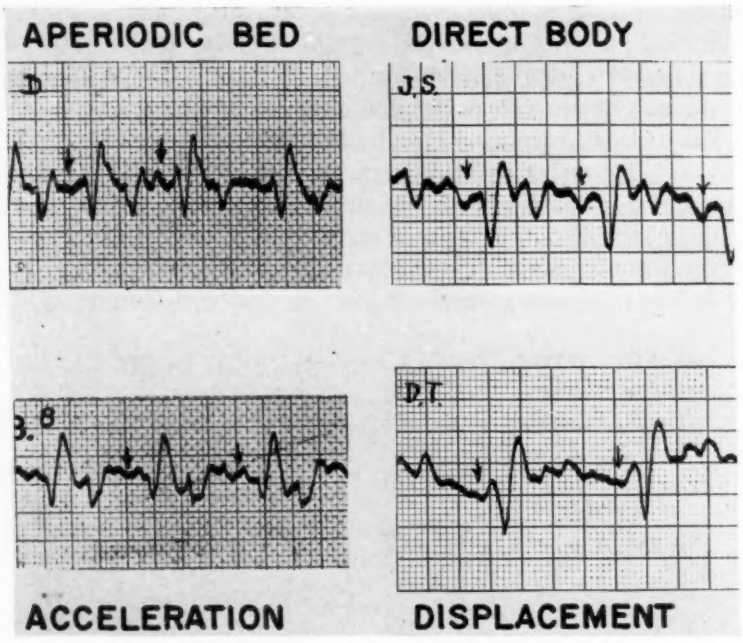


Fig. 3.—Presents again the marked similarity between traces taken from the aperiodic bed and the direct displacement body technique when the feet and shoulders were packed with sandbags. Again, the arrows indicate the onset of the QRS complex. Tracings on the left are taken from records published in the Bull. Johns Hopkins Hosp. (Talbot and associates[2]) and represent normal ballistocardiograms from subjects on a platform floating on mercury (the function of body motion being recorded with an accelerometer). Tracings on the right are direct damped body ballistocardiograms recorded with displacement technique. Again note the marked similarity between the two records, both in configuration and time relationship.

METHOD

It is generally accepted that the body as it lies on a rigid surface acts as a mass on a spring having a particular resonant frequency, responding to the applied circulatory forces in a manner that is modified by the relationship of the forcing frequency to that resonant frequency, and to the degree of damping that is present. Regarded as such a mechanical system, it then may be considered according to the physical laws governing vibratory systems and their responses to external or driving forces. The differential equations that may be applied to the determination of amplitude and phase shift response relative to the frequency of the applied force for a given vibratory system are a matter of basic mechanical physics. Burger has published equations for the determination of amplitude and phase response relative to the displacement of the center of gravity of the circulatory mass and viscera, as applied to the low-frequency bed. In this instance, we are concerned with such response relative to the circulatory force as applied to the direct body technique when measured both as displacement and as acceleration. The equations and their derivation used here may be found in the Appendix. The variables that must be known to solve such equations are: (1) the natural or resonant frequency of the system involved—in this instance, of the body; (2) the ratio of the actual damping present in the system to critical damping; (3) the frequencies of the applied force. By solving these equations for a number of arbitrarily selected frequencies of applied force, a curve or graph may be plotted which will show the frequency response characteristics of the system. Since the frequency response is actually a function of the ratio between the resonant frequency and the applied or forcing frequencing, the basic frequency response curves were plotted against such a ratio with the specific frequencies of the different systems to be considered extrapolated along the abscissa of the graph.

DETERMINATION OF VARIABLES

The natural frequency of the body, as it lies on a rigid surface, has been shown previously from this laboratory to range from 3 to 7 cycles per second (c.p.s.).[7] The resonant frequency of the body in the putty bed packed with sandbags was reported by Tucker and Ostrom[7] to be approximately 8 c.p.s. Since these figures were from a small number of subjects, it seemed desirable to restudy a larger group of individuals by both direct body techniques, first recording the ballistocardiogram from the subjects as they lay on a rigid surface without additional damping, and from the same subjects by the putty bed modification as described by Tucker and Ostrom.[7] The natural frequency and damping factors were experimentally determined in 17 subjects, ranging in age from 20 to 64 years, in the following manner:

A. *Undamped Direct Body Techniques.*—The subjects were recumbent on a concrete floor without head or foot rest. Displacement was sensed from the head by the bellows air-conduction system described previously.[6] The recorder was a Sanborn Poly-viso Cardiette. The natural frequency of the body was determined by imparting a headward push to the heel of the subject during diastole. The force of the push was so adjusted as to cause a deflection in the general range of amplitudes of the largest ballistocardiographic waves, so as not to exceed the elastic limit of the system. The oscillations due to the natural body frequency were separated readily from the ballistocardiographic complexes and easily measured, and the resonant frequency calculated. The damping factor was determined by measuring the initial deflection and the subsequent overshoot beyond the base line, and applying the equation as described by Burger and co-workers[4] for the determination of the delta or damping value (See Appendix).

B. *Putty Bed Technique.*—Similar maneuvers were carried out on the same subjects as they lay on a bed of putty packed with sandbags, as described previously.[7] Sensing and recording were accomplished with the same instruments as in the unmodified technique.

From these data frequency response curves for both amplitude and phase angle were calculated for both direct body techniques.

RESULTS

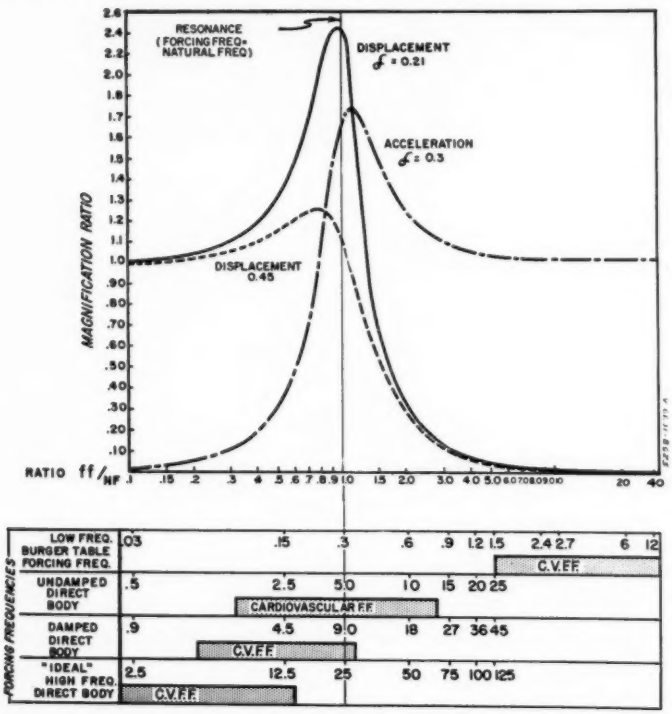
The natural frequencies and damping factors, as determined for the two direct body techniques, are shown in Table I. It may be seen that the effect

TABLE I

	UNDAMPED DIRECT BODY TECHNIQUE		PUTTY BED TECHNIQUE	
SUBJECT	NATURAL FREQUENCY	OVERSHOOT RATIO	NATURAL FREQUENCY	OVERSHOOT RATIO
D. T.	5.5	0.39	7.1	0.17
W. S.	6.2	0.40	8.2	0.29
E. K.	4.6	0.51	7.0	0.26
J. R.	4.7	0.47	8.7	0.29
M. H.	5.6	0.58	8.5	0.34
McN.	4.9	0.57	7.9	0.26
J. P.M.	6.4	0.50	9.3	0.18
J. W.	5.5	0.53	8.9	0.23
J. S.	5.0	0.53	8.5	0.18
F. S.	5.4	0.71	9.4	0.16
D. R.	4.9	0.61	8.2	0.09
J. M.	5.3	0.48	9.4	0.12
B. L.	5.1	0.34	7.7	0.11
L. H.	4.4	0.60	6.5	0.24
J. V. A.	5.5	0.63	8.4	0.26
M. N.	5.3	0.46	7.7	0.19
V. C.	4.5	0.45	8.7	0.23
Average	4.93	5.13 = 0.21	8.1	2.06 = 0.45

of the putty bed was to increase body frequency, and to increase the damping toward critical. This effect was seen in every instance.

The natural frequencies from the undamped direct body technique were found to range from 4.4 to 6.4 c.p.s., with a mean value of 4.93 c.p.s. from



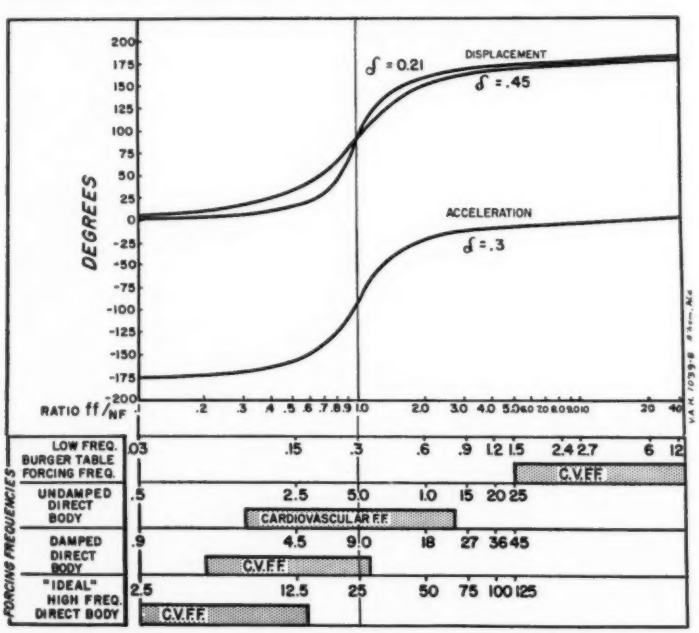
Graph I.—The amplitude response curve when recording displacement or acceleration and its relationship to the ratio of the forcing frequency to the natural frequency. The magnification ratio (vertical axis) indicates the amplitude response with the different systems. A magnification ratio of 1 represents a true linear response in any system. On the horizontal axis, a ratio of 1 is the point at which the cardiovascular forcing frequencies which are generating the ballistocardiographic waves are equal to the resonant frequency of the body or the system by which the ballistocardiogram is taken. An increase in the ratio indicates the cardiovascular forcing frequencies are higher than those of the natural frequencies. Similarly, a decrease in the ratio indicates the forcing frequency is below the natural frequency. The solid line represents the response from the usual *displacement* system which is essentially undamped (damping factor of 0.21). The dash line represents a displacement response curve in which the damping is approximately doubled with a damping factor of 0.45. The dot-dash curve represents the response when the motion is measured as acceleration with a damping factor of 0.3. The table below the graph represents a scheme for the forcing frequency as they apply to this graph and is similarly presented in Graph II for four different types of systems. The dotted blocks in the lower chart represent an assumed range of cardiovascular forcing frequencies. Recent evidence indicates that higher forcing frequencies may be present, especially in myocardial disease. First is the low-frequency Burger table, or aperiodic type suspension, in which the resonance of the body system is 0.3 and therefore resonance distortion is only noticed when the forcing frequencies are 0.3 c.p.s. This is probably the ideal system since the curve actually extends out beyond the represented range and higher cardiovascular forcing frequencies can be recorded than by other systems. Note that with the undamped direct body type of technique, there is marked distortion at the resonance range or when the forcing frequencies are equal to the natural frequency (approximately 5 c.p.s.). Thus, the magnification distortion of the record is probably in the center of the cardiovascular forcing frequencies as indicated by the dotted block. Since body frequency is almost doubled by damping the body with sandbags applied to the feet and shoulders, most of the cardiovascular frequency forces are now to the left of the resonance range or on a relatively linear aspect of the displacement response curve with a damping factor of 0.45. Note that this curve is only linear in its responses to the point where the ratio of the forcing frequencies or natural frequencies are just above that of resonance. This seriously limits the damped direct body technique as the only forces adequately registered are those which are just at and below the resonance (in this instance, approximately 9 to 13 c.p.s.). The fourth dotted block represents an ideal high-frequency direct body technique. This can be achieved only if the natural body resonance could be increased to approximately 25 c.p.s. Then the cardiovascular forcing frequency would lie on a relatively linear aspect of the displacement curve with a damping factor of 0.45.

the 17 subjects studied. In the putty bed, the resonant frequencies were found to range from 6.5 c.p.s. to 9.4 c.p.s., with a mean value of 8.1 c.p.s. The increased damping of the putty bed is illustrated by the decrease in overshoot after the induced diastolic deflection. The overshoot ranged from 34 per cent to 74 per cent of the initial deflection when the subject lay on the floor, whereas in the putty bed this overshoot range was found to be 9 to 34 per cent. The delta value, or the ratio of the actual damping to critical damping, was found to increase from an average of 0.21 of critical damping on the floor to an average of 0.45 of critical in the putty. Graph I is a plot of the amplitude response relative to a given force, so that a response which is directly proportional to the *force* and is unmodified by factors of body resonance will be expressed as unity on the ordinate, against the frequencies of the applied forces on the abscissa. Curves are plotted for displacement for both of the determined mean delta values and for acceleration at a delta value of 0.3, which is that of the Burger low-frequency bed and is midway between the determined values for both direct systems. It is readily apparent that when displacement is recorded from any system, there will be a uniform, "accurate" response relative to force only when the resonant frequency is significantly higher than the frequence of the applied force, or when the ratio of the forcing frequency to resonant frequency is considerably less than one. As the applied or driving force approaches the resonant frequency of the system, there will be an exaggeration of the amplitude response to that force. When the resonant frequency is passed or the forcing frequencies become higher than the resonant frequency, there will be a sharp roll-off of the amplitude response, so that when the forcing frequency is approximately twice that of the resonant frequency, the response will be extremely small to the relative applied force. Conversely, when acceleration is recorded, there will be a uniform response directly proportional to the applied force only for those forces whose frequencies are significantly higher than the resonant frequency of the ballistic system. Again there is a marked increased amplitude at the resonant range, with a rapid roll-off in amplitude as the forcing frequencies become less than the resonant frequency of the system. The importance of the damping factor is illustrated by the difference in the two curves, representing *displacement* at different degrees of damping factors. It is readily apparent that as the damping factor is increased toward critical damping, there is a marked diminution in the tendency of the amplitude response to increase beyond that which is proportional to the force as the resonant range is approached. There is also a considerable decline in the rapidity of the roll-off as the resonant frequency is crossed.

From this study, it would appear that the major forces of the normal circulation range from 2 c.p.s. to approximately 12 c.p.s. The frequency spectrum of the circulatory forces is illustrated by the crosshatched bar for each of the ballistic systems shown in Graph I. When this frequency spectrum is compared to the amplitude response curves, the reasons for the similarity

between the acceleration traces from the low-frequency system and the displacement traces from the putty bed (delta 0.4) become apparent, since this frequency spectrum of the force fails across the undistorted response section of the frequency response curves for both systems. The reasons for the differences between these two systems and the undamped direct body technique (delta 0.2) also are apparent, since in the latter method the frequency spectrum of the cardiovascular forces producing the ballistocardiogram lies directly across the resonant frequency of that system, with an exaggerated response of those frequencies occurring at the resonant range and sharp diminution of the amplitude response of those frequencies which lie above the resonant range. The limitations of the putty bed technique are also clearly demonstrated. For the mean figures of resonant frequency and damping obtained, it is apparent that the latter system will give a relatively undistorted response (plus or minus 20 per cent) for those cardiovascular forces having a frequency below 12 c.p.s. The low-frequency systems give an undistorted response for forcing frequencies above 0.3 c.p.s.

Graph II illustrates the phase (time relations) response curves, in the same manner as the frequency response curves are illustrated in Graph I, for

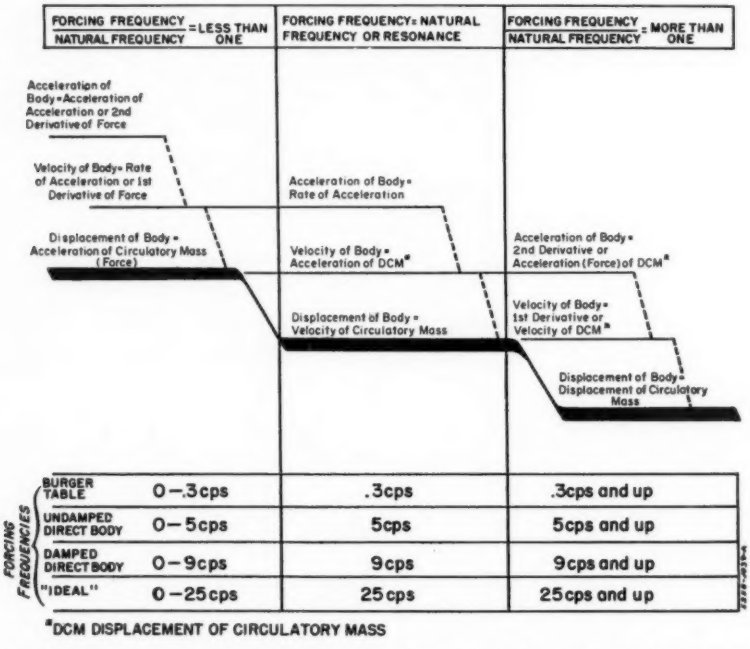


Graph II. The phase response curve as related to the ratio of forcing frequency to natural frequency. Note that displacement is in phase with force (approximately 0 degrees) only for the frequencies *below* the ratio of resonant frequency. In other words, when recording with displacement, only those frequencies which are below the natural frequency of the system (5 in the undamped and 9 in the damped direct body) will be in phase with the applied force. At body resonance, there is an approximate 90-degree phase shift, and above resonance, the phase shift reaches approximately 180 degrees. If the motion is being recorded with acceleration, it is in phase only when the forces are *above* the body frequency or the resonance of the system. The effect of the various degrees of damping on the curves are included in the damping factors for the various lines. The table below the graph relates examples of the various BCG systems to the phase response curves. Each of the frequencies in the table apply to the ratio immediately above it. For example, a ratio of 1 would correspond to a forcing frequency of 5 c.p.s. in the undamped system, 9 in the damped system, 0.3 for the Burger suspension.

the phase response or time relations of the various ballistic systems when recorded as displacement and acceleration. It will be observed that the general contour of the curves is quite similar to the amplitude response, with the exception that invariably at the resonant range there will be a 90-degree phase shift, and that the displacement and acceleration curves maintain a constant 180-degree relationship. From the correlation of the cardiovascular force (frequency spectrum), which is again indicated by the crosshatched bars, with the frequency response curves, several important observations may be made: (1) When acceleration is recorded from the low-frequency system, the recorded deflections are in phase with the entire spectrum of the cardiovascular forces. (2) When displacement is recorded from the putty bed, the recorded deflections or displacements are in phase with those cardiovascular forces below the range of resonance, and at resonance (for this system, 8.3 c.p.s.) there is a 90-degree phase lag which progresses toward a 180-degree phase lag for the highest end of the assumed spectrum. (3) When acceleration is recorded from the direct body systems, only those cardiovascular forces significantly above resonance will be in phase with the recorded deflections. At resonance and below, there will be a phase difference of 90 degrees and 180 degrees, respectively. These angles are negative, indicating a phase lead rather than a phase lag, so that the recorded deflections of the acceleration traces from the putty bed for forces having a frequency of 3 to 5 c.p.s. are 360 degrees away from the same forces recorded as displacement from the low-frequency systems.

Thus it is apparent that for the assumed frequency spectrum of cardiovascular forces, the displacement ballistocardiogram from the putty bed is in fact a force ballistocardiogram, and is comparable to acceleration records of the low-frequency system. A force ballistocardiogram is the second derivative of the displacement ballistocardiogram from a low-frequency system. If acceleration (second derivative of displacement) were recorded from the putty bed system, then the recorded deviations would be proportional to the fourth derivative of displacement of the circulatory mass, or the second derivative of its force. A similar trace from the aperiodic system could be obtained by measuring the acceleration of an accelerometer. The functions of motion as described are "pure" only when the cardiovascular forcing frequencies are within the range specified. Therefore, when the resonant frequency lies within the range of forcing frequencies such as in the undamped direct body systems, the resulting traces will be a mixture of all functions of motion (Fig. 4). Similarly, if there are forces above the assumed frequency spectrum, then the ballistocardiogram recorded as displacement from the putty bed will be a mixture of the primitive (displacement of circulatory mass) for the higher frequencies; its first derivative at the resonant range, and its second derivative for those frequencies below resonance. If acceleration were recorded from this system, the tracings would again be a mixture, in this instance of the second, third, and fourth derivatives of displacement of the circulatory mass. These relationships are illustrated in Fig. 4. Since such a mixture of

functions must exaggerate the complexity of analysis, it is evident that the practical application of all techniques must be considered in the light of the frequencies of the forces to be studied. As mentioned previously, the major frequency of cardiovascular forces in the normal subject may be from 1 to 12 c.p.s., which is the assumed spectrum illustrated in the graphs in this report. However, other studies by Rappaport,[8] Arbeit and associates,[9] and Talbot and Harrison[10] have suggested that forces of higher frequencies may be present both in normals, and more particularly, in abnormals. However, the significant question that must be answered for this purpose is whether such forces are of great enough energy to be registered by a system having a linear and


FORCING FREQUENCY / NATURAL FREQUENCY = LESS THAN ONE
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Acceleration of Body = Acceleration of Acceleration or 2nd Derivative of Force
Velocity of Body = Rate of Acceleration or 1st Derivative of Force
Acceleration of Body = Rate of Acceleration
Displacement of Body = Acceleration of Circulatory Mass (Force)
Velocity of Body = Acceleration of DCM*
Acceleration of Body = 2nd Derivative or Acceleration (Force) of DCM*
Displacement of Body = Velocity of Circulatory Mass
Velocity of Body = 1st Derivative or Velocity of DCM*
Displacement of Body = Displacement of Circulatory Mass
FORCING FREQUENCIES
BURGER TABLE 0–.3cps .3cps .3cps and up
UNDAMPED DIRECT BODY 0–5cps 5cps 5cps and up
DAMPED DIRECT BODY 0–9cps 9cps 9cps and up
"IDEAL" 0–25cps 25cps 25cps and up
*DCM DISPLACEMENT OF CIRCULATORY MASS

Fig. 4.—Diagram shows the function of motion which is obtained when recording displacement, velocity, and acceleration from the various systems. The first column represents the response when the ratio of the forcing frequency to natural frequency is less than one. The range of the assumed forcing frequencies for the various systems is noted at the bottom of the graph. Note that from the Burger table, all cardiovascular forces, from 0 to 0.3 c.p.s., when recorded with an accelerometer, will be registered in effect as the second derivative of force. Similarly, by recording velocity from an ultra-low-frequency system, such as the Burger table, all forces below 0.3 c.p.s. will be represented as the rate of acceleration or the first derivative of force. Since cardiovascular force frequencies below 0.3 c.p.s. seem unlikely, this is of no significance. Note that below body resonance (0 to 9 c.p.s.) with the damped direct technique, all forces below 9 c.p.s. must be recorded with displacement in order to represent acceleration of the circulatory mass or force ballistocardiogram. Similarly, if one records acceleration from this system, all forces below body resonance (5 c.p.c. undamped, 9 c.p.s. damped) will be registered as the second derivative of force or the acceleration of acceleration. The second column represents the function of motion which is registered when forcing frequency is equal to the natural frequency or resonance. Note that with the direct body techniques, forces (5 and 9 c.p.s., respectively) will be registered as displacement when recording the velocity of body motion. Similarly, if they are recorded as velocity, they will be registered as acceleration of circulatory mass or force traces, and when the body is recorded with an accelerometer, they will be registered as the rate of acceleration or the first derivative of acceleration. The last column represents the response where the ratio of the forcing frequencies to the natural frequencies is more than one. Recording acceleration of the body would now be equal to the acceleration or second derivative of displacement of circulatory mass or force ballistocardiogram. Thus in the low-frequency or aperiodic systems recording the motion with an accelerometer results in a trace which is in phase with the acceleration of the circulatory mass. In the direct technique, only the forces above resonance will be similarly registered, while the forces below resonance will be recorded as first and second derivatives of the displacement of the circulatory mass.

flat response. Fourier analysis is difficult to perform and to interpret with confidence when the wave forms are as complex as with the ballistocardiograph. In addition, the BCG forces are transient rather than constant, and therefore, may not be determined accurately by a simple Fourier analysis. The frequency response curves for amplitude and phase having been calculated, and having determined values for such factors from young, normal subjects by the three different systems, it should be possible to determine the major frequencies of the force vectors resulting in the individual ballistic deflections within certain limits by comparing the changes induced by the different techniques and correlating them with the frequency response curves. For example, if a given ballistocardiogram deflection is of greater amplitude in the putty bed than from the floor and yet is at the same relative amplitude by both the putty bed and the mercury bed, it is apparent that such a deflection was produced by a forcing frequency beyond the resonant range of the first system but below the resonant range of the second. Additional information may be obtained from the phase response curves in a similar manner. The results of this study enable one to reach some tentative conclusions as to the most important frequencies encountered in normal subjects: (1) Since the very slight increase in frequency response induced by the putty bed causes a major change in the BCG, as compared to the undamped direct body technique, there must be significant forces at or about those frequencies. (2) Since there is a relative decrease in amplitude of some of the waves (J-K) by the putty bed and also by the low-frequency techniques, it may be inferred that such deflections are at the resonant range of the undamped direct body, or approximately 5 c.p.s. (3) Since there are few, if any, deflections of even minor amplitude seen in the records published by Talbot and associates from the aperiodic bed that are not also seen by the putty bed, admitting amplitude differences, it would appear that the significant forces in normal subjects are within the recording range of the putty bed, or below approximately 12 c.p.s.

It is evident that these comparisons were on normal subjects, and it does not necessarily follow that the frequencies encountered in such individuals will encompass the range of significant frequencies in abnormal states. In fact, recent experience suggests that the direct body systems will not give an accurate representation of the forces associated with diseased myocardium. It is also acknowledged that these figures were taken from subjects with relatively slow heart rates, and it is apparent that at least part of the frequencies encountered will be related to the heart rate, though probably not in a linear fashion. It is clear, however, that in normal subjects with relatively slow rates the forces concerned with the ballistocardiograms are very close to the frequency of the undamped body and are at or below the frequency of the damped body in the putty bed.

DISCUSSION

The observation that displacement BCG's from a relatively high-frequency system have striking similarity to acceleration BCG's from a very low-frequency system, and the explanation of such similarity have several points of

interest. In the first place, it is a laboratory demonstration of the accuracy of the physical principles described by Burger and associates.[4] A second point of interest in the similarity of the displacement and acceleration "force BCG's" is that it appears to be strong evidence that both techniques have largely eliminated the distortion produced by the external elastic tissues of the body. This contention is further supported by the observation that Schwarzschild[11] has obtained almost identical "force BCG's" by electronically canceling out some of the influences of body elasticity without altering the actual motion of the body as it lies on a rigid surface.

The absolute accuracy of the putty bed, or any high-frequency system yet devised, appears to be definitely limited. As regards the putty bed, it must be admitted that its very narrow linear frequency response may be a drawback. As previously mentioned, appreciation of its ultimate value will depend upon a more precise definition of the frequencies encountered in disease states. Even if one were willing to accept less than linear amplitude responses, the disturbing possibility of phase shift (time distortion) would remain and, as shown, the putty bed has an even more limited linear range for phase response than amplitude. A 90-degree phase shift would imply time lag of one quarter of the period of a cycle, while a 180-degree shift would mean that a deflection would lag the force by one half the period of that force. Such distortion would obviously make physiologic correlation extremely uncertain. Even in normals, the evidence presented would suggest some distortion of phase and amplitude. There are various ways to further raise body frequency even more than by the putty bed. Further experimentation with such techniques is now in progress.

The putty bed does offer an intriguing aspect in studying first and second derivatives of force. Since force is recorded by displacement from the putty bed, then one can record the first and second derivatives of acceleration or force with readily available displacement, velocity, and acceleration transducers, recognizing the previously mentioned possibility of mixing of such derivatives in a single record. Such mixing is obviously present in the low-frequency, undamped direct body techniques used by Smith and Bryan,[12] and Arbeit and Lindner,[13] and others who have studied velocity and acceleration BCG's from such systems. The effect of such differentiation would be to progressively increase the amplitude of the higher frequency components with a decrease of the deflections resulting from the lower frequency forces, as shown in Graph I. The result would be an exaggeration of the abnormalities of those disease states characterized by increased high-frequency forces, and to minimize the abnormalities of the BCG's of those states characterized by increased force in the lower frequency ranges. The clinical value of such derivation remains to be established by comparing such traces with the undifferentiated force traces in various disease states, but it would appear that such traces would complement the force BCG's by increasing their clinical sensitivity. From this one technique, one might obtain relatively undistorted

force BCG's, with such distortion that is present tending to exaggerate low-frequency activity, and simultaneously obtain the first and second derivatives of force to exaggerate minimal changes of diseases characterized by high-frequency activity. Arbeit and co-workers,[9] and Smith and associates[14] already have suggested the potential value of such selective distortion to exaggerate BCG abnormality of specific disease states, although their studies have been with the undamped direct body techniques. While it is true that similar information might be obtained by recording from an accelerometer of an accelerometer from an aperiodic system, such instruments are not readily available, and the possible difficulties of application are not known. Electronic derivation of the acceleration tracings of an aperiodic system is possible but difficult, and is fraught with possibilities of gross distortion.

SUMMARY

1. It has been shown that acceleration BCG's from aperiodic or very low-frequency ballistocardiograms are similar, in young normal subjects, to the displacement BCG's from a direct body technique altered so as to increase body frequency and the degree of damping of the system.

2. The resonant frequencies and ratios of damping of young normal subjects were determined for an unmodified direct body technique and for the putty bed technique. The effect of the putty bed and packing was to increase the resonant frequency and damping coefficient in all subjects.

3. Frequency response curves for amplitude and phase relationship were plotted from the data obtained, for an undamped direct body method, and for the putty bed modification of the direct body techniques. When these curves were compared to the frequency response curves of acceleration of a low-frequency system, the reasons for similarity of ballistocardiograms from the two systems through a limited range of forcing frequencies became apparent.

4. It was shown that the velocity and acceleration traces from an ideal high-frequency ballistocardiogram would be proportional to the third and fourth derivatives of the displacement of the circulatory mass, or to the first and second derivatives of the acceleration or force of the circulatory mass.

5. Potential application of such derivations was briefly discussed.

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APPENDIX

If it is assumed that the body is a simple harmonic oscillator driven by a force having its origin in the heart, the differential equation describing the motion of the body then is:

$$m \frac{d^2x}{dt^2} + \beta \frac{dx}{dt} + kx = F e^{j\omega t} \tag{1}$$

where m is body mass; β is the velocity dependent friction coefficient; k is the spring constant; F is the amplitude of the driving force; and x is the instantaneous displacement of the body. The sinusoidal nature of the driving force is represented by $e^{j\omega t}$ where $j = \sqrt{-1}$ and ω equals $2\pi f$, f being the driving force frequency.

A solution of the above differential equation will yield the displacement, x, as some function of time, t. Ideally, this solution would be the sum of two separate solutions—the transient solution and the steady state solution.[15] With the amount of damping present in ballistocardiography, the complete solution will rapidly approach the steady state solution, therefore the transient solution is at present omitted.

A simple method[16] can be used to find the steady state solution. A sinusoidal function of undetermined phase and amplitude, but of frequency equal to that of the driving force, is substituted for the displacement x in the differential equation (1) and the indicated differentiation carried out. Thus letting

$$x = Xe^{j\omega t}$$

where X is the complex amplitude coefficient of our solution, containing also the phase, and substituting in (1) gives

$$X\,(-m\omega^1 + j\beta\omega + k)\, e^{j\omega t} = Fe^{j\omega t},$$

or

$$X = \frac{F/m}{\left(\omega_o^2 - \omega^2 + j\,\beta\,\frac{\omega}{m}\right)}.$$

Since $\omega_o^2 = \frac{k}{m}$ (2)

If we use δ to represent the ratio of β to its value at critical damping, then

$$X = \frac{F/m}{(\omega_o^2 - \omega^2 + 2\delta\,\omega_o\omega)}. \tag{3}$$

Substituting

$$A = \omega_o^2 - \omega^2 \text{ and } B = 2\delta\omega_o\omega,$$

$$X = \frac{F/m}{A + jB}.$$

Multiplication by the complex conjugate then gives

$$X = \frac{F/m\ (A - jB)}{A^2 + B^2}.$$

Substituting this value of X in $x = Xe^{j\omega t}$, and using the relation

$$e^{j\omega t} = \cos \omega t + j \sin \omega t$$

we obtain

$$x = \frac{F}{m}\frac{(A - jB)}{(A^2 + B^2)} (\cos \omega t + j \sin \omega t).$$

If we multiply and take the real part, then

$$x = \frac{F}{m\ (A^2 + B^2)} (A \cos \omega t + B \sin \omega t),$$

which is equivalent to

$$x = \frac{F}{m\ \sqrt{A^2 + B^2}} \cos\ (\omega t + \phi),$$

where $\phi = - \tan^{-1} \frac{B}{A}$;

$$\text{or } x = \frac{F}{m\sqrt{\omega_o^2 - \omega^2) + 4\delta^2\omega^2\omega_o^2}} \cos\ (\omega t + \phi), \tag{4}$$

where

$$\phi = - \tan^{-1} \frac{2\ \delta\ \omega\omega_o}{\omega_o^2 - \omega^2}. \tag{5}$$

ϕ is the phase of the displacement with respect to the driving force. A negative ϕ indicates displacement lags the force.

A more convenient form of equation (4) is

$$x = \frac{F}{m\omega^2 \sqrt{\left(1 - \frac{\omega^2}{\omega^2}\right)^2 + 4\delta^2 \frac{\omega^2}{\omega_o^2}}} \cos\ (\omega t + \phi). \tag{6}$$

The amplitude of the displacement is

$$X = \frac{F}{m\omega^2 \sqrt{\left(1 - \frac{\omega^2}{\omega_o^2}\right)^2 + 4\delta^2 \frac{\omega^2}{\omega_o^2}}}$$

or, since $k = m\omega_o^2$,

$$X = \frac{F}{k \sqrt{\left(1 - \frac{\omega^2}{\omega_o^2}\right)^2 + 4\delta^2 \frac{\omega^2}{\omega_o^2}}}.$$

The ratio $\frac{X}{\frac{F}{k}} = \frac{1}{\sqrt{\left(1 - \frac{\omega^2}{\omega_o^2}\right)^2 + 4\delta^2 \frac{\omega^2}{\omega_o^2}}}$

is known as the magnification ratio. It is the ratio of the dynamic displacement per unit force to the static displacement per unit force. Graph I shows the magnification ratio vs. frequency for various degrees of damping. Graph II shows the corresponding phase shifts.

The acceleration can be obtained by differentiating equation (6) twice, yielding

$$a = \frac{d^2x}{dA^2} = \frac{F}{\frac{m\omega_o^2}{\omega^2}\sqrt{\left(1 - \frac{\omega^2}{\omega_o^2}\right)^2 + 4\delta^2\frac{\omega^2}{\omega_o^2}}}\cos\ (\omega t + \phi). \tag{7}$$

From this can be obtained a ratio

$$\frac{a/F}{1/m} = \frac{-1}{\frac{\omega_o^2}{\omega^2}\sqrt{\left(1 - \frac{\omega^2}{\omega_o^2}\right)^2 + 4\delta^2\frac{\omega^2}{\omega_o^2}}}\cos\ (\omega t + \phi). \tag{8}$$

which represents the amplitude of the acceleration per unit force obtained during oscillation to the acceleration per unit force which would be obtained in a periodic rectilinear motion; that is, without resonance effects. This is shown graphically in Graph I. The phase of the acceleration is altered by the minus sign since

$$-\cos\ (\omega t + \phi) = \cos\ (\omega t + \phi - 180°).$$

the acceleration lags the displacement by 180°.

$$\frac{A/F}{1/m} = \frac{1}{\frac{\omega_o^2}{\omega^2}\sqrt{\left(1 - \frac{\omega^2}{\omega_o^2}\right)^2 + 4\delta^2\frac{\omega^2}{\omega_o^2}}}\cos\ (\omega t + \sigma), \tag{9}$$

where $\sigma = \phi - 180°$ is the phase angle of acceleration with respect to force. Phase lag of acceleration with force is shown in Graph II.

INHIBITION OF BIOLOGIC ACETYLATION BY 1-HYDRAZINOPHTHALAZINE

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THE hypotensive drug 1-hydrazinophthalazine* (hydralazine, Apresoline C-5968) has been widely used in the management of hypertension. A number of investigators have reported that the administration of this substance over relatively long periods of time and in relatively high doses was associated with late side effects simulating some of the rheumatic diseases. The features of the "hydralazine syndrome" have been reviewed recently.[1] Perry and associates[2, 3] have studied some of the metabolic and chemical properties of this compound.

The present investigations were designed to study the effects of 1-HP in systems capable of enzymatic acetylation in vitro. Crude pigeon liver extracts were employed to bring about the acetylation of sulfanilamide, D-glucosamine, and hydroxylamine in the presence of various concentrations of 1-HP. The effects of the drug on rates of acetylation of the various compounds were noted. Other studies were made on the behavior of 1-HP when it was treated with the liver extracts in the presence of appropriate cofactors.

MATERIALS AND METHODS

Pigeon liver extracts were prepared by the method of Kaplan and Lipmann.[4] When the acetylation of sulfanilamide was studied, reaction mixtures containing sulfanilamide, sodium acetate, ATP, sodium citrate, TRIS buffer, cysteine hydrochloride, CoA, and liver extract were prepared according to Govier and Gibbons.[5] 1-HP was added to the mixtures in various concentrations. The total volume used in the incubation was 1.3 ml. The mixtures were incubated for one hour at 37° C. with air as the gas phase and without shaking. Free sulfanilamide in TCA filtrates of the mixtures was determined by the Bratton and Marshall procedure.[6] When glucosamine acetylation was studied, the procedure of Chou and Soodak[7] was used. N-Acetylglucosamine formed during the incubation was determined in the TCA filtrates by the Chou and Soodak modification[7] of the Morgan and Elson method.[8] The assays of the "acetate-activating" system of the extract were carried out by the hydroxamic acid method of Chou and Lipmann.[9] The components of the reaction mixtures were the same as those used by Seaman,[10] except that cysteine replaced glutathione, and were used in the same molar proportions but in twice the concentrations. The acethydroxamic

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This work was supported in part by a grant from Winthrop Laboratories, 1450 Broadway, New York 18, N. Y. After this manuscript had been prepared, a report by Johnson and Corte (Proc. Soc. Exper. Biol. & Med. 92: 446, 1956) appeared in which it was reported that 1-hydrazinophthalazine inhibited the acetylation of Isoniazid.

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*Abbreviations used in this paper include 1-HP, 1-hydrazinophthalazine; ATP, adenosine triphosphate; TRIS, tris-(hydroxymethyl)-aminomethane; CoA, coenzyme A; TCA, trichloroacetic acid; AMP, adenosine monophosphate; PP, pyrophosphate. We wish to thank Dr. William E. Wagner, Ciba Pharmaceutical Products, Inc., Summit, N. J., for a generous gift of crystalline 1-HP.

acid formed was measured by the Lipmann and Tuttle method.[11] In experiments in which the time-course of the disappearance of 1-HP was studied, 0.5 μM of the drug was incubated with the mixture used above,[5] except that sulfanilamide was omitted. The concentration of 1-HP remaining in the tubes at the end of various incubation periods was determined by a modification of the method of Perry.[12] To 4.5 ml. of 0.4 M acetate buffer, pH 4.0, was added 0.5 ml. of TCA filtrates of the incubation mixtures. To this, 0.5 ml. of 0.24 per cent ninhydrin in isopropyl alcohol was added and the mixture heated for 7 minutes at 70° C. The yellow color formed was determined in a Klett-Summerson photoelectric colorimeter using the 42 filter. The instrument was adjusted to zero with a blank containing all the components except 1-HP. In experiments in which the requirements for disappearance of the drug were investigated, the same mixture[5] was used with the exception that both citrate and sulfanilamide were omitted.

RESULTS

The effects of 1-HP on the acetylation of sulfanilamide are shown in Table I. In these experiments, 2 units of CoA per tube were used. Since the quantity of sulfanilamide used here was 0.4 μM per tube, it is apparent that the molar ratio of 1-HP to sulfanilamide necessary to cause 50 per cent inhibition is approximately 1.3. When this ratio was increased to approximately 8, the reaction was inhibited by 95 per cent.

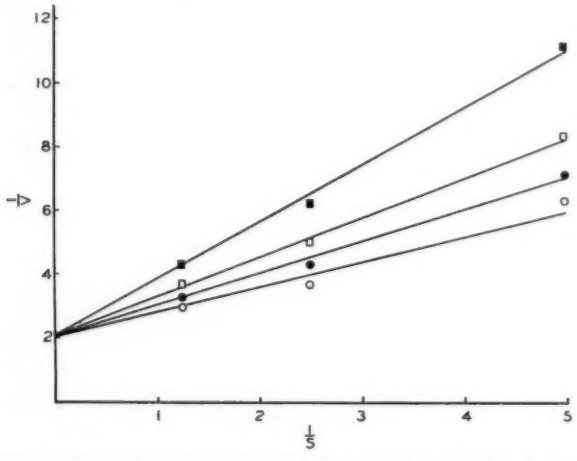

Fig. 1.—Competitive inhibition of acetylation of sulfanilamide by 1-HP. The velocity (v) is expressed as μM sulfanilamide actylated per hour. The substrate concentration (s) is expressed as μM sulfanilamide per tube. Open circles, 0 μM 1-HP; closed circles, 0.25 μM 1-HP; open squares, 0.5 μM 1-HP; closed squares, 1.0 μM 1-HP.

TABLE I. INHIBITION OF SULFANILAMIDE ACETYLATION BY 1-HP

μM 1-HP/TUBE	PER CENT INHIBITION
0.5	51.3
1.0	77.3
3.0	94.6

Fig. 1 is a plot of the reciprocal velocities versus the reciprocal substrate concentrations in a series of experiments which were carried out in the presence of various concentrations of 1-HP. It is seen that the Lineweaver and Burk postulate[13] for competitive inhibition is fulfilled, that is, the curves intersect the ordinate at a point.

In the experiment on glucosamine acetylation, it was found that 3.0 μM of 1-HP per tube inhibited the reaction by 92 per cent.

Table II summarizes the results of experiments on the formation of acethydroxamic acid from acetate and hydroxylamine in the presence of several concentrations of 1-HP. It is seen that increasing concentrations of the drug are without effect here.

TABLE II. INFLUENCE OF 1-HP ON FORMATION OF ACETHYDROXAMIC ACID

μM OF 1-HP/TUBE	UNITS OF CoA/TUBE	μM OF ACETHYDROXAMIC ACID FORMED
0	6	0.29
1	20	0.29
3	20	0.22
5	20	0.22
28	6	0.31

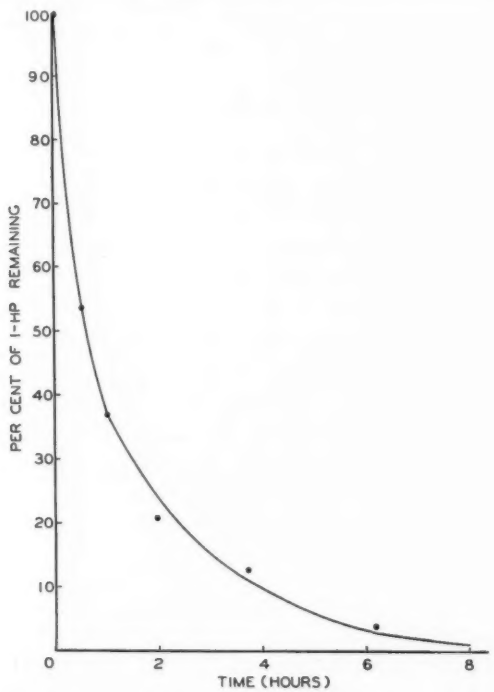

Fig. 2—Disappearance of 1-HP from incubation mixture as a function of time.

That 1-HP disappears from the reaction mixtures with time is shown in Fig. 2. One-half of the initial amount of the compound disappears in approximately 30 minutes, and it completely disappears in 8 hours. The influence of ATP, CoA, and acetate on the disappearance of 1-HP (1 μM) from mixtures of cysteine hydrochloride, TRIS buffer, and dialyzed pigeon liver extract are shown in Table III. Preliminary studies had shown that the crude extracts retained a relatively high endogenous capacity to metabolize the drug. In order to study the requirements for this metabolism, it was necessary to lower this endogenous capacity by dialysis for 18 hours at 4° C. against a large volume

TABLE III. THE EFFECTS OF ADDITIONS OF ATP, CoA, ACETATE, AND COMBINATIONS ON THE DISAPPEARANCE OF 1-HP

ADDITIONS	μM OF 1-HP METABOLIZED/5.5 HR.
None	0.11
Acetate	0.12
ATP	0.06
CoA	0.00
ATP + CoA	0.00
Acetate & CoA	0.00
ATP + acetate	0.05
ATP + acetate + CoA	0.28

of distilled water. This treatment caused a rather appreciable loss in activity of the enzymes in the extract and necessitated a longer incubation period than was used in the case of the sulfanilamide acetylation. The figures in Table III represent the drug metabolized in five and one-half hours. The dialysis lowered the endogenous activity to such an extent that the simultaneous addition of ATP, CoA, and acetate caused a significant increase in the amount of drug disappearing.

DISCUSSION

The work of Defranceschi and Zamboni,[14] and Makino and co-workers[15] on the metabolism of isonicotinic acid hydrazide and the work of Johnson[16] on several aromatic and heterocyclic hydrazides have demonstrated that acetylation represents a significant metabolic fate of these compounds. It therefore seems reasonable that hydrazines might undergo the same transformation. In the experiments reported here, we have shown that the inhibition of the acetylation of sulfanilamide by 1-HP in the pigeon liver extracts is competitive. One mechanism for such a relationship is the competition between the two substances for "active acetate" in the form of acetyl-CoA. Such a mechanism would account for the inhibitory effect of 1-HP on the acetylation of glucosamine. Chou and Lipmann[9] have established that the process by which biologic acetylations occur is as follows:

1. Activation of acetate:

$$\text{acetate} + \text{CoA-SH} + \text{ATP} \rightarrow \text{acetyl-S-CoA} + \text{AMP} + \text{PP}$$

2. Transfer of acetyl group:

$$\text{acetyl-S-CoA} + \text{HX} \rightarrow \text{acetyl} - \text{X} + \text{CoA-SH}$$

where HX represents the substrate being acetylated.

The first reaction may be studied by measuring the rate of formation of acethydroxamic acid in the presence of hydroxylamine. When we assayed the effect of 1-HP on this reaction, it was found to be without influence (Table II), lending further weight to the assumption that the drug was exerting its effect in the second reaction. From Fig. 2 it is seen that incubation of the drug in the reaction mixture in the absence of sulfanilamide results in its disappearance —0.5 μM being completely metabolized in 8 hours. It is also seen (Table III) that a combination of ATP, acetate, and CoA appreciably enhances the disappearance of the drug over any of them separately or in combinations of two. This further argues for acetylation as a metabolic fate of 1-HP.

To our knowledge the basic mechanism of the pharmacologic action of 1-HP is unknown. Further investigation of its effects on acetate metabolism might yield information of consequence regarding its action as a hypotensive agent and also in regard to the metabolic abnormalities associated with the "hydralazine syndrome" and spontaneous and experimental rheumatic diseases.

SUMMARY

1-Hydrazinophthalazine has been found to inhibit the acetylation of sulfanilamide and glucosamine in pigeon liver extracts. This inhibition is of a competitive nature and appears to be the result of the drug being acetylated by acetyl-CoA.

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STUDIES ON THE INCIDENCE AND DISTRIBUTION OF ANTIBIOTIC-RESISTANT STAPHYLOCOCCI

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THE incidence and treatment of staphylococcal infections have been markedly influenced in recent years by a series of changes in the distribution and antibiotic resistance of the causal agent. These changes, without doubt, must be attributed to the extensive, and at times unjustified, use of antibacterial drugs. In the recent literature many workers have made the general observation that a direct relationship exists between the resistance rate and the antibiotic in current favor.[1, 2]

An assessment of the validity of this observation was made possible as a result of striking changes in the amounts of the antibiotics used in the Kingston General Hospital during the past three years. Data collected from the hospital pharmacy are recorded in Table I and indicate that whereas the amounts of penicillin and the tetracyclines dispensed during the study period have fallen to a significant degree, there has been a marked increase in the use of chloramphenicol and erythromycin.

TABLE I. AMOUNTS OF ANTIBIOTICS USED IN THE KINGSTON GENERAL HOSPITAL 1953 TO 1955

ANTIBIOTIC	AMOUNT OF ANTIBIOTIC IN GRAMS		
	1953	1954	1955
Penicillin	10,000	8,000	6,000
Total tetracyclines	4,000	5,000	1,800
Chloramphenicol	2,200	3,600	10,800
Erythromycin	300	600	3,000

Considering these changes in therapeutic practice, it was thought to be of value to study their possible effect on the antibiotic sensitivity and distribution of coagulase-positive staphylococci in this area.

METHOD

A total of 1,326 strains of staphylococci were isolated from infected inpatients and outpatients during the period January, 1954, to April, 1956. Isolates were made from human blood agar plates and coagulase activity determined by the slide technique. Sensitivity tests were carried out by the medicated disc method using discs in the following concentrations, penicillin 1.5 units, tetracycline 10 μg, chloramphenicol 10 μg, and erythromycin 10 μg. A sterile swab, dipped in an 18-hour broth culture of the strain to be tested, was evenly

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smeared over the surface of a dried nutrient agar plate. The discs were placed on the inoculated plate and gently pressed flat on the agar. After overnight incubation at 37° C. staphylococci which showed a zone of inhibition of any size were regarded as sensitive.

Nasal cultures were obtained from 217 Queen's University students who did not have any remarkable hospital contact, and from 222 nurses and orderlies who were continuously exposed to the hospital environment. Nasal swabs were streaked on Difco Staphylococcus Medium 110 and several colonies subcultured after 48 hours of incubation at 37° C. Coagulase tests and sensitivity tests then were carried out on the pure subcultures. The density of the nasal population was arbitrarily graded from ++++ to 0 on the basis of a count of the number of colonies of coagulase-positive staphylococci which appeared on the plates.

The distribution of staphylococci in the dust of the hospital was assessed by exposing plates of Difco Staphylococcus Medium 110 for a period of 1 to 2 hours in the rooms and throughout the corridors. One hundred fifty-four strains of coagulase-positive staphylococci were isolated, no more than one strain was picked from any one exposure plate. Nasal and dust cultures all were taken during April, 1956.

RESULTS

Antibiotic-resistant staphylococci were found most commonly in those groups of strains associated directly with hospital environment (Tables II and III), i.e., strains from inpatients, hospital staff, and hospital dust. Outpatient strains reflected the general character of the hospital strains but contained an impressive number of sensitive strains. The organisms isolated from the university students were preponderantly sensitive to all four drugs. The three groups of hospital strains appeared closely related; the common strains sensitive to all four drugs, resistant to penicillin alone, and resistant to penicillin and tetracycline only.

TABLE II. A COMPARISON OF THE ANTIBIOTIC SENSITIVITY OF COAGULASE-POSITIVE STAPHYLOCOCCI ISOLATED FROM DIFFERENT GROUPS

GROUP	NO. OF STRAINS TESTED	PER CENT RESISTANT TO:			
		PENICILLIN	TETRACYCLINE	CHLORAMPHENICOL	ERYTHROMYCIN
Students	93	6.5	2.2	0.0	0.0
Outpatients	272	22.1	15.1	1.9	1.5
Hospital dust	154	48.1	24.0	9.7	4.5
Inpatients	1054	62.9	48.3	10.8	11.7
Hospital staff	108	77.8	35.2	10.2	1.9

TABLE III. A COMPARISON OF THE SENSITIVITY PATTERNS OF COAGULASE-POSITIVE STAPHYLOCOCCI ISOLATED FROM DIFFERENT GROUPS

Penicillin	S*	R†	R	R	S	R	R	S	R	S	R	S	S	R
Tetracycline	S	R	S	R	R	R	S	S	R	R	S	R	S	S
Chloramphenicol	S	S	S	R	S	S	S	S	R	R	R	S	R	R
Erythromycin	S	S	S	S	S	R	R	R	R	S	S	R	S	R
Students	91.4‡	0.0	6.5	0	2.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Outpatients	72.1	9.6	10.7	0.7	4.8	0.0	0.7	0.4	0.0	0.0	0.0	0.0	0.7	0.4
Hospital dust	46.1	14.3	24.7	4.5	1.3	2.0	0.0	0.6	0.0	0.6	3.2	1.3	0.6	0.6
Inpatients	24.6	27.9	19.8	5.2	6.5	4.6	3.6	1.8	1.1	2.6	0.6	0.5	1.2	0.1
Hospital staff	19.4	30.6	38.9	2.8	0.0	0.9	0.0	0.0	0.9	0.0	3.7	0.0	2.8	0.0

*S = Sensitive.
†R = Resistant.
‡ = Per cent of strains showing this pattern.

The difference between the resistance of staphylococci carried by the hospital staff and the students was very striking; 80.6 per cent resistant as opposed to 8.6 per cent resistant. Although no significant difference was noted

in the carrier rate between these two groups ($p = 0.23$) the hospital employees were shown to have a much heavier nasal population of staphylococci than the students (Table IV).

TABLE IV. A COMPARISON OF THE NASAL POPULATION OF COAGULASE-POSITIVE STAPHYLOCOCCI IN STUDENTS AND NURSES

	TOTAL NO. TESTED	NO. CARRYING COAG. POS. STAPHYLOCOCCI	NO. OF CARRIERS WITH A STAPHYLOCOCCAL NASAL POPULATION OF			
			++++	+++	++	+
Students	217	93 (42.8%)	6 (6.5%)	7 (7.4%)	22 (23.6%)	58 (62.4%)
Hospital staff	222	108 (48.7%)	25 (23.1%)	17 (15.7%)	29 (26.9%)	37 (34.3%)

A general comparison of the antibiotic sensitivity of the strains of coagulase-positive staphylococci isolated during 1954 and 1955 is indicated in Table V. A decrease in the number of strains isolated resistant to penicillin and tetracycline and an increase in the number of chloramphenicol- and erythromycin-resistant strains has occurred during this period. The change is seen in the strains isolated from patients in hospital and is reflected to an even more marked degree in the outpatients' strains. A consideration of Table I would suggest that these variations in sensitivity are directly related to the amount of the various antibiotics dispensed during the study period.

TABLE V. A COMPARISON OF THE ANTIBIOTIC RESISTANCE OF COAGULASE-POSITIVE STAPHYLOCOCCI ISOLATED FROM INFECTED PATIENTS IN 1954 AND 1955

GROUP	NO. OF STRAINS TESTED	PER CENT RESISTANT TO:			
		PENICILLIN	TETRACYCLINE	CHLORAMPHENICOL	ERYTHROMYCIN
Inpatients 1954	474	69.0	51.9	8.4	9.9
Inpatients 1955	580	57.9	45.4	12.8	13.1
Outpatients 1954	117	33.3	23.1	0.9	0.0
Outpatients 1955	155	12.9	9.7	1.1	1.1

The case of erythromycin is a special one. This drug was used only sparingly during 1954, but during the first half of 1955 it was extensively prescribed for the treatment of a variety of infections. Following the publication of several reports[3-5] which indicated the rapid emergence of resistant strains during promiscuous use of erythromycin, the Kingston General Hospital staff thought it wise to restrict the use of this agent to the treatment of infections due to penicillin-resistant staphylococci. The institution of this restriction was followed by a 75 per cent reduction in the amount of erythromycin dispensed during the last half of 1955.

Table VI indicates that the number of resistant strains isolated directly parallels the amount of drug used, progressively increasing as the use of chloramphenicol increases and fluctuating with the varying use of erythromycin.

TABLE VI. RELATIONSHIP BETWEEN THE RESISTANCE OF STAPHYLOCOCCI TO ERYTHROMYCIN AND CHLORAMPHENICOL AND THE AMOUNT OF DRUG USED

PERIOD OF STUDY	NO. OF STRAINS TESTED	RESISTANT TO ERYTHROMYCIN		AMOUNT OF ERYTHROMYCIN USED IN GM. PER MONTH	RESISTANT TO CHLORAMPHENICOL		AMOUNT OF CHLORAMPHENICOL USED IN GM. PER MONTH
		NO.	PER CENT		NO.	PER CENT	
1954*	474	47	9.92	50	40	8.44	300
1955A†	271	43	15.86	400	28	10.33	800
1955B‡	309	33	10.68	100	46	14.99	1000

*1954—strains collected from January, 1954, to January, 1955.
†1955A—strains collected from January, 1955, to July, 1955.
‡1955B—strains collected from August, 1955, to April, 1956.

DISCUSSION

The above results would appear to justify the conclusion that the number of antibiotic-resistant strains of staphylococci isolated from a semiclosed community is directly related to the amount of a given antibiotic administered within that community.

The extensive reviews of Finland[1] and Wise and associates[2] support this observation and indicate that without exception the emergence of staphylococci resistant to a given drug has followed a constant pattern. When the drug is first introduced only a very few resistant strains are observed, unless a related drug has been in considerable use and the phenomenon of cross-resistance is in operation between the old and the new drug. With increasing availability of the antibiotic, the number of resistant strains isolated from hospital populations is seen to increase. Less frequent use of the drug is associated with a decrease in the number of resistant strains. Pathogenic staphylococci carried by the general population remain predominantly sensitive.[6-8]

The mechanics of this pattern of emergence of resistant strains and the ultimate distribution of these strains is of great interest, and the following is postulated as a possible sequence of events.

Staphylococci in the dust derived from clothing and bedclothes of carriers are undoubtedly important in the environmental transfer of these organisms.[9] Organisms carried on the skin are also important in the direct transfer of infection[10] and in the pathogenesis of staphylococcal skin lesions. However, there is little doubt that the primary reservoir of coagulase-positive staphylococci in nature is in the human nasopharynx.[11, 12]

This reservoir is not only a massive one, involving as it does some 25 to 50 per cent of the normal population at any one time, but also a fluctuating one. Lepper and co-workers[13] have demonstrated a fairly rapid turnover of specific strains. They found that, in as short a period as one month, two-thirds of the carriers studied by this group lost their specific nasopharyngeal strains, with different strains being implanted in these or other members of the population, at a similar rate.

In a semiclosed community, such as a hospital, the elimination of antibiotic-sensitive staphylococci from the nasopharynges of patients being treated with antibacterial agents exerts a distributional pressure on the strains being

passed among patients and hospital personnel, resulting in the implantation of a higher proportion of resistant strains. This would tend to bring about a marked increase in the number of carriers of resistant strains among treated patients[14] and these strains would then be introduced into the cycle of strains being transferred among the staff members. Time, of course, can be shown to play an important part. The longer a person is treated with antibiotics or is in contact with the hospital environment, the greater the chance of implantation of a resistant strain.[15]

Over a period of time one would expect the accumulation in the hospital staff of a hard core of carriers of resistant strains; a situation further aggravated by the presence of persistent carriers who constitute some 10 to 20 per cent of the population.[13] These tend to retain a specific strain for long periods and once a resistant variant is established in these carriers they become a most important and persistent source for the transference of these strains to other staff members and patients.

Further increasing the rate of transfer of resistant strains is the demonstration by many workers of an absolute increase in the carrier rate in hospital staff members.[16] Although this was not conclusively shown in our study, it was apparent that the staphylococcal nasal flora of the hospital personnel was much heavier than that of the control group. Thus it seems likely that a person who enters a hospital environment will be subject to the onslaught of a heavy population of resistant organisms.

Variations in the number of resistant strains isolated will depend in part on the selective pressure induced by the amount of antibiotic used within a given institution. The more antibiotic used, the more the implantation of resistant strains is favored. The less antibiotic used the less the number of carriers available for selective implantation of resistant strains and the more sensitive strains may be re-established from the reservoir in the normal population.

Resistant strains carried by members of the general population usually are implanted during treatment with antibiotics and/or hospitalization. These strains may be transferred to close contacts, and although the mass of sensitive strains carried in the general population tends to decrease the probability of implantation of a resistant strain, a widespread and injudicious use of antibiotics outside of the hospital could have a significant effect on the population of resistant organisms.[8]

Many other factors are undoubtedly operating in the transfer of resistant staphylococci aside from the ones mentioned above, and the problem of control is a very difficult one. However, this problem might be approached from several points of view.

1. The prevention of cross-infection in patients by dust control procedures and by rigid aseptic technique in the operating room and in the dressing of wounds.

2. The prevention of emergence of resistant strains by limiting the amount of antibiotic used both in and out of the hospital. This limitation, of course, can be applied only within the demands of sound and legitimate use of these

drugs in treatment and prophylaxis. That this limitation is effective is well shown above by our experience with erythromycin. It is certainly clear that a strenuous effort should be made to prevent the administration of antibiotics in the absence of specific indications. This is particularly true of the prophylactic use of antibiotics, a use which is in urgent need of very careful reassessment.[17-19]

The amount by which antibiotic consumption needs to be reduced in order to produce a significant fall in the number of resistant strains may not be as great as one would at first envisage. One may liken the situation to the control of other biologic balances such as epidemics of infectious disease. Students of epidemiology are well aware of the fact that in order to prevent large outbreaks of disease it is not necessary to maintain a completely immune population or to eliminate completely a parasite from the environment. So long as sanitary control and immunization can maintain the number of cases of infection below a certain threshold for that disease, epidemics are not likely to occur.

Similarly, if we consider the action of antibiotics to be the creation of a susceptible population, i.e., susceptible to the implantation of resistant strains, then it well may be that a relatively minor reduction in the amount of antibiotics used—still within the demands of sound and legitimate therapeutic and prophylactic use—may lower the threshold typical of this balance sufficiently to achieve a major reduction in the number of carriers of resistant strains.

SUMMARY

1. The antibiotic resistance and distribution of coagulase-positive staphylococci isolated from five different sources has been studied.

2. The staphylococcal nasal flora of the hospital staff is shown to be much heavier than that of persons having no hospital contact although the absolute carrier rate is not significantly different.

3. The strains isolated from different sources fall into the following order of increasing number of resistant isolates: University students 8.6 per cent; hospital outpatients 29.9 per cent; hospital dust 53.9 per cent; hospital inpatients 75.4 per cent; hospital staff 80.6 per cent. A close interrelationship exists among the strains isolated from the last three groups.

4. During the period January, 1954, to April, 1956, the number of strains isolated resistant to penicillin and tetracycline decreased and the number resistant to chloramphenicol and erythromycin increased. This change is shown to be directly related to the amount of the various antibiotics administered in the Kingston General Hospital during this period.

5. The limitation of the use of erythromycin is shown to be an effective method of controlling the rate of emergence of strains resistant to this drug.

6. The mechanism of the emergence of resistant strains and their distribution is discussed.

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EXPERIMENTAL STUDIES ON BACTERIAL CONTAMINATION OF BANK BLOOD

III. The Survival and Growth of Bacteria in Bank Blood Incubated at 37° C. for Short Periods Before Refrigeration

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MANY investigators have concluded that a certain small percentage of bank bloods will become contaminated with bacteria during collection in spite of the most rigid safeguards and precautions taken.[1-3] Whitby[1] estimated that the average contamination rate in British blood banks during the war years (1939-1945) was approximately 5 per cent, while Heath and Amgren[4] during the same period found that 8.5 per cent of 6,151 L. of plasma collected in New York City contained bacteria. More recently, Braude and his colleagues[3] reported that 2.24 per cent of 1,697 bloods collected in a routine manner at a university blood bank was contaminated.

While it is generally true that most fresh whole bloods are able to kill or suppress the growth of a wide variety of bacteria, the earlier premise that, ". . . contamination, if present, could not propagate, and consequently, would be so attenuated as to be completely innocuous"[5] has been amply disproved by the number of serious or fatal reactions due to heavily contaminated blood which have been reported in recent years.[6-12] In addition to these catastrophic reactions, it seems probable that some of the relatively mild "pyrogenic" reactions which are of much more frequent occurrence may be due to small numbers of bacteria in the transfused blood. Landy and Johnson[13] have shown that the endotoxin released from 10,000 typhoid organisms after intravenous injection is sufficient to cause a pyrogenic response in rabbits. This number of bacteria in a pint of blood would represent a concentration of only 17 organisms per milliliter and Braude and associates[3] have reported that the concentration of bacteria found in contaminated blood twenty-four hours after collection was 14 cells per milliliter.

Many different antiseptic agents and dyes have been added to bank blood in order to prevent bacterial contamination, but none were completely satisfactory.[14-18] The potential hazard involved in injecting these agents intravenously into already debilitated patients also was influential in deciding against their use. Recent investigations with antibiotics[2, 19] appear more promising, but additional studies are necessary to determine whether the addition of these

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agents to bank blood is effective and entirely safe. During the course of our previous studies on bank blood contamination,[20] it was observed that the numbers of viable coliform bacteria inoculated into fresh blood dropped sharply when the blood was allowed to remain at room temperature for one hour prior to refrigeration. This finding suggested the possibility that the natural antibacterial activity of fresh whole blood might be considerably enhanced by incubating the blood at 37° C. for brief periods before the blood was stored at refrigerator temperature. After preliminary investigations revealed that short periods of incubation at 37° C. had no apparent deleterious effects on fresh red cells, studies were carried out on the survival and propagation of various types of gram-negative bacteria in such incubated bloods in order to determine whether this procedure offered a practical means of reducing the amount of bank blood contamination.

METHODS AND MATERIALS

Blood.—The blood used in this study was generously made available by the Irwin Memorial Blood Bank of the San Francisco County Medical Society. It was procured* and processed in the usual fashion and received in this laboratory unrefrigerated within 2 hours after collection from the donor. The bloods obtained were from both sexes and of various blood types, but were not satisfactory for routine use because of positive Kahn tests, lipemia, etc. A total of 27 different units of blood was employed in this investigation.

Bacteria.—The gram-negative bacteria were divided into 2 groups on the basis of their growth characteristics in broth at 4° and 37° C. and are listed in Table I. We are indebted to Dr. A. I. Braude, Southwestern Medical School, Dallas, Texas, Dr. Margaret Pittman, National Institutes of Health, Bethesda, Maryland, and Dr. Alcor S. Browne, California State Department of Public Health, Berkeley, California, for providing us with cultures. Most of the cold growing organisms kindly furnished by Drs. Pittman and Braude had been isolated from contaminated blood or blood bank rooms and, in some cases, had been implicated in transfusion reactions. Stock cultures were grown in Proteose Peptone No. 3 broth (Difco) for 24 hours and diluted to the desired concentration in sterile saline immediately before inoculation into the blood.

Procedure of testing.—The blood, immediately upon arrival, was aseptically dispensed in 49 ml. amounts into large rubber-stoppered test tubes and warmed in a 37° C. waterbath for 20 minutes before inoculation. Control blood sterility cultures were performed at this time by spreading 0.2 to 0.3 ml. of whole blood onto duplicate agar plates and incubating 1 plate at room temperature and 1 plate at 37° C. for 2 weeks.

In the experiments with Group I "warm-growing" bacteria, 1 ml. of the diluted bacterial culture was inoculated into duplicate blood tubes and mixed, and 1 tube was placed in the refrigerator at 4° C. to serve as a control, while the second tube was returned to the 37° C. water bath for various periods of time. Both sets of tubes were gently agitated occasionally during this initial period to insure thorough mixing of bacteria and blood elements. After the incubation period, the experimental tubes were placed in the refrigerator alongside the control tubes and all subsequent manipulations were carried out in parallel with both sets of tubes. In the experiments with Group II "cold-growing" bacteria, hundred fold dilutions of each bacterial culture were inoculated into each of three 19 ml. blood aliquots in order to obtain a final concentration of approximately 10^6, 10^4 and 10^2 cells per millimeter of whole blood. One tube of each set was immediately placed in the refrigerator as a control, while the second and third tubes of each set were returned to the water bath for 1 and 3

*The bloods were collected by the vacuum method in glass bottles containing 120 ml. of Acid-Citrate-Dextrose solution as anticoagulant and red cell preservative per 480 ml. of whole blood.

TABLE I. TYPES AND GROWTH CHARACTERISTICS OF BACTERIA USED IN THIS STUDY

ORGANISM-STRAIN	SOURCE
GROUP I: RAPID GROWTH AT 37° C., SLOWER GROWTH AT 22° C., NO GROWTH AT 4° C.	
Escherichia coli (Me) *Escherichia coli* (Ty) *Escherichia coli* (Pe) *Escherichia coli* (Da) *Escherichia coli* (Ho) *Escherichia coli* (1)	Univ. of Calif. Dept. of Microbiology stock cultures
Pseudomonas aeruginosa (Co) *Pseudomonas aeruginosa* (Ko) *Pseudomonas aeruginosa* (Mu) *Pseudomonas aeruginosa* (Ti) *Pseudomonas aeruginosa* (Ek) *Pseudomonas aeruginosa* (2) *Pseudomonas aeruginosa* (4)	Univ. of Calif. Dept. of Microbiology stock cultures
Aerobacter aerogenes (1) *Aerobacter aerogenes* (3) *Aerobacter aerogenes* (11) *Aerobacter aerogenes* (24) *Aerobacter aerogenes* (Ca)	Univ. of Calif. Dept. of Microbiology stock cultures
Paracolobactrum—Bethesda, 0 group 3 *Paracolobactrum*—Bethesda, 0 group 18 *Paracolobactrum*—Arizona, type 5-17, 20 *Paracolobactrum*—Arizona, type 7-1, 7, 8	Dr. Alcor S. Browne, Calif. State Dept. of Public Health, Berkeley, Calif.
Paracolobactrum intermedium (2)	Univ. of Calif.—isolated from contaminated blood
Proteus vulgaris (OX19)	Univ. of Calif. stock culture
Serratia marcoscens (1)	Univ. of Calif. stock culture
GROUP II: RAPID GROWTH AT 22° C., GOOD GROWTH AT 4° C., GOOD TO POOR GROWTH AT 37° C.	
Pseudomonas—IB20A *Pseudomonas*—IB14B	Dr. A. I. Braude
Pseudomonas—PC117 *Pseudomonas*—PC136, F3	Dr. M. Pittman
Pseudomonas—IMBB5	Univ. of Calif.—isolated from contaminated blood
Achromobacter—IB12A *Achromobacter*—IB13A *Achromobacter*—IB28 *Achromobacter*—IB81	Dr. A. I. Braude
Aerobacter cloacae—PC140 *Aerobacter cloacae*—PC189	Dr. M. Pittman
Paracolobactrum aerogenoides—PC119B	Dr. M. Pittman
Escherichia intermedium—IMBB4	Univ. of Calif.—isolated from contaminated blood

hours, respectively, before they were stored with the control in the refrigerator. Estimations of the ability of the inoculated cultures to survive and propagate in the blood samples were made 1, 7, 14, and 21 days after inoculation by spreading 0.1 ml. of whole blood, or appropriate dilutions, onto duplicate agar plates with a bent glass rod. Care was taken to reduce to a minimum the amount of time the blood samples were exposed to room temperature. If the concentration of cold-growing bacteria in a particular tube reached 10^8 cells per milliliter before the end of the 21-day test period, the counts from that tube were discontinued, since previous studies had shown that such cultures would persist at this level

for long periods of time. The agar plates made from blood containing warm-growing bacteria were incubated at 37° C. for 24 hours and at room temperature for 24 hours before colony counts were made. The agar plates containing the cold-growing organisms were incubated at room temperature for 72 hours before counts were made.

RESULTS

Studies With Warm-Growing Bacteria.—The majority of bacterial contaminants which gain access to bank blood during collection are coagulase-negative staphylococci, diphtheroids, and gram-negative organisms of the enteric group.[2, 21] The majority of these contaminants in both groups are unable to multiply at low temperatures and the gram-positive organisms have only rarely been implicated in serious transfusion reactions.[11] In the experiments reported here, 6 different species, comprising 25 strains, of common gram-negative bacteria were tested for their ability to survive and propagate in 20 different bloods incubated at 37° C. for various periods of time before the bloods were refrigerated at 4° C. With the exception of one strain of paracolon bacteria,* none of the strains tested were capable of significant multiplication in blood at refrigerator temperature over the 21-day period. When these organisms were inoculated into broth and stored at 4° C., most of the strains survived or showed only a gradual reduction in numbers during the test period. Therefore, the difference in numbers of viable bacteria remaining in the experimental and control tubes 24 hours after inoculation was, in the essence, a measure of the bactericidal power of the blood alone under the conditions tested. The size of the inoculum used (10^4 to 10^6 cells per milliliter of whole blood) in most of the experiments was greater than that which might be expected to occur under natural circumstances,[3] but it was necessary to use such numbers in order to demonstrate clear-cut differences. We have arbitrarily chosen a 2 log or greater difference in bacterial concentration between the experimental and control tubes as being significant and, for convenience, only the data of the first and twenty-first day after inoculation are reported.

From the results obtained in this portion of the study and summarized in Table II, it seemed apparent that incubation of most bank bloods at 37° C. for 2 to 4 hours significantly enhanced the killing or suppressive properties for many of the bacterial strains tested. Only 4 of the 20 different units of blood used failed to show any such enhancement. Since an individual blood was usually tested against strains of only 1 or 2 species, the failure to obtain increased killing power of the 4 bloods above may simply be a reflection of the type of organism used in the tests. The 16 bloods which did manifest increased killing power after incubation reduced the number of viable bacteria in the experimental tubes by at least 2 logs, and in some cases by 4 logs, in 31 of the 99 experiments conducted. Although the preliminary incubation of the bloods would appear to favor the initiation of bacterial growth, no significant multiplication could be detected in the incubated blood samples 24 hours after inoculation, even in those cases where the blood apparently lacked any suppressive

*_Paracolobactrum intermedium_[2] was not able to multiply in broth at 4° C., but fairly rapid growth, beginning 7 to 14 days after inoculation, was sporadically observed in some bloods stored at 4° C.

TABLE II. THE INFLUENCE OF INCUBATION OF FRESH BLOOD AT 37° C. FOR BRIEF PERIODS BEFORE REFRIGERATION ON THE SURVIVAL OF GRAM-NEGATIVE BACTERIA

ORGANISM	NO. OF STRAINS TESTED	NO. OF DIFFERENT BLOODS TESTED	AVERAGE NO. OF BACTERIA INOCULATED PER MILLILITER OF WHOLE BLOOD	INCUBATION TIME AT 37° C. BEFORE REFRIGERATION	NO. OF EXPERIMENTS OVER TOTAL WITH 2 LOG OR GREATER REDUCTION IN BACTERIAL CONCENTRATION IN INCUBATED BLOOD SAMPLE		NO. OF EXPERIMENTS OVER TOTAL IN WHICH BLOOD WAS STERILE OR CONTAINED LESS THAN 10 BACTERIA PER MILLILITER BY END OF 21 DAYS	
				HOURS	DAY 1	DAY 21	INCUB.	CONTROL
Escherichia coli	6	8	10^4	2	8/28	6/28	18/28	13/28
Pseudomonas aeruginosa	7	6	10^4	2.5	11/23	12/23	10/23	1/23
Paracolobactrum	5	8	10^6	3	5/28	8/28	16/28	6/28
Aerobacter aerogenes	5	4	10^6	3.5	4/16	0/16	11/16	9/16
Proteus vulgaris	1	2	10^4	4	2/2	2/2	1/2	0/2
Serratia marcescens	1	2	10^4	4	1/2	2/2	0/2	0/2
Total					31/99	30/99	56/99	29/99

factors for the strain tested. However, in two experiments the refrigerated control blood effected a greater initial reduction in the bacterial inoculum than did the incubated sample.

The differences found between the numbers of bacteria in the experimental and control tubes at the end of the 21-day test period in general paralleled those observed at the end of the first day. This was to be expected, since the organisms in both bloods were exposed to similar environmental conditions between the first and twenty-first day after inoculation. Nevertheless, in 7 of the 30 tests where significant differences occurred at the end of 21 days, no such differences were apparent on the first day after inoculation. This has been interpreted as a delayed inhibitory effect on the microorganisms in the incubated blood samples. At the end of the test period, 56 out of the 99 incubated bloods were sterile or contained less than 10 bacteria per milliliter, whereas only 29 of the 99 controls were similarly free of organisms. This is a highly significant difference, $p = <0.001$. The incubated bloods generally contained fewer viable bacteria 1 day after inoculation than the controls and the length of survival in the blood was apparently related to the numbers of organisms present at this time.

That there may be marked variations in the ability of different bloods to kill or suppress the growth of a particular type or strain of bacteria is not surprising. Some of the bactericidal properties of the blood are undoubtedly related to past exposure of the host, the blood donor, to the various microorganisms. Other bactericidal properties, e.g., properdin levels, may also vary.[22]

This variability in antibacterial activity is illustrated by two typical experiments shown in Tables III and IV, in which 1 blood was tested against 5 different types of gram-negative bacteria, while another was tested against 5 strains of the same organism. These results clearly show that no faith can be placed in the ability of any given blood to eliminate the particular chance contaminants that may gain access to bank blood during collection.

TABLE III. BACTERICIDAL PROPERTIES OF ONE BANK BLOOD FOR FIVE SPECIES OF GRAM-NEGATIVE BACTERIA

ORGANISM	NO. OF BACTERIA INOCULATED PER MILLILITER OF WHOLE BLOOD	NUMBER OF VIABLE BACTERIA PER MILLILITER OF WHOLE BLOOD			
		DAYS AFTER INOCULATION			
		1	7	14	21
A. aerogenes (1)	6×10^4	0	0	0	0
P. intermedium (2)	4×10^4	2×10^2	<10	0	0
E. coli (1)	4×10^4	1×10^3	7×10^2	1×10^2	3×10^2
S. Marcescens	1×10^5	3×10^4	3×10^5	2×10^4	3×10^3
P. vulgaris (OX19)	5×10^4	2×10^4	2×10^4	6×10^1	4×10^4

TABLE IV. BACTERICIDAL PROPERTIES OF ONE BANK BLOOD FOR FIVE STRAINS OF *E. Coli*

STRAIN OF *E. coli* INOCULATED	NO. OF BACTERIA INOCULATED PER MILLILITER OF WHOLE BLOOD	NUMBER OF VIABLE BACTERIA PER MILLILITER OF WHOLE BLOOD			
		DAYS AFTER INOCULATION			
		1	7	14	21
Me	2×10^4	0	0	0	0
Ty	3×10^4	0	0	0	0
Da	8×10^4	6×10^2	2×10^2	7×10^1	2×10^2
Pe	1×10^4	1×10^4	8×10^3	2×10^3	4×10^2
He	1×10^4	1×10^4	2×10^3	2×10^2	2×10^1

Studies With Cold-Growing Bacteria.—In this portion of the investigation the organisms tested were capable of relatively rapid multiplication in broth at 4° C. The methods employed to determine survival and propagation of the organisms in the incubated and control blood samples were modified somewhat in order to observe the concomitant effects of both the size of the bacterial inoculum and the period of incubation at 37° C. A total of 7 different bloods and 5 different types of cold-growing bacteria were tested as follows: 5 Pseudomonas strains versus 5 bloods, 4 Achromobacter strains versus 4 bloods, 2 Aerobacter strains versus 3 bloods, 1 Paracolobactrum strain versus 2 bloods, and 1 Escherichia strain versus 1 blood.

The enhancement of bactericidal activity of incubated blood previously found against warm-growing bacteria was found also in many instances with the cold-growing organisms. In some experiments preincubation reduced the numbers of bacteria to very low levels, but only rarely sterilized the blood. The results of representative experiments with 4 different types of organisms in 4 different bloods are shown graphically in Fig. 1. After initial suppression of the bacterial inoculum, the few survivors usually were able to initiate relatively rapid growth and eventually reached high concentrations during the 21-day test period. With inocula of 10^4 to 10^6 bacteria per milliliter, the

numbers of organisms present in the incubated bloods after one day were generally significantly lower than in the controls, but when growth in the former tubes began the rate of growth in both samples was roughly parallel. By the

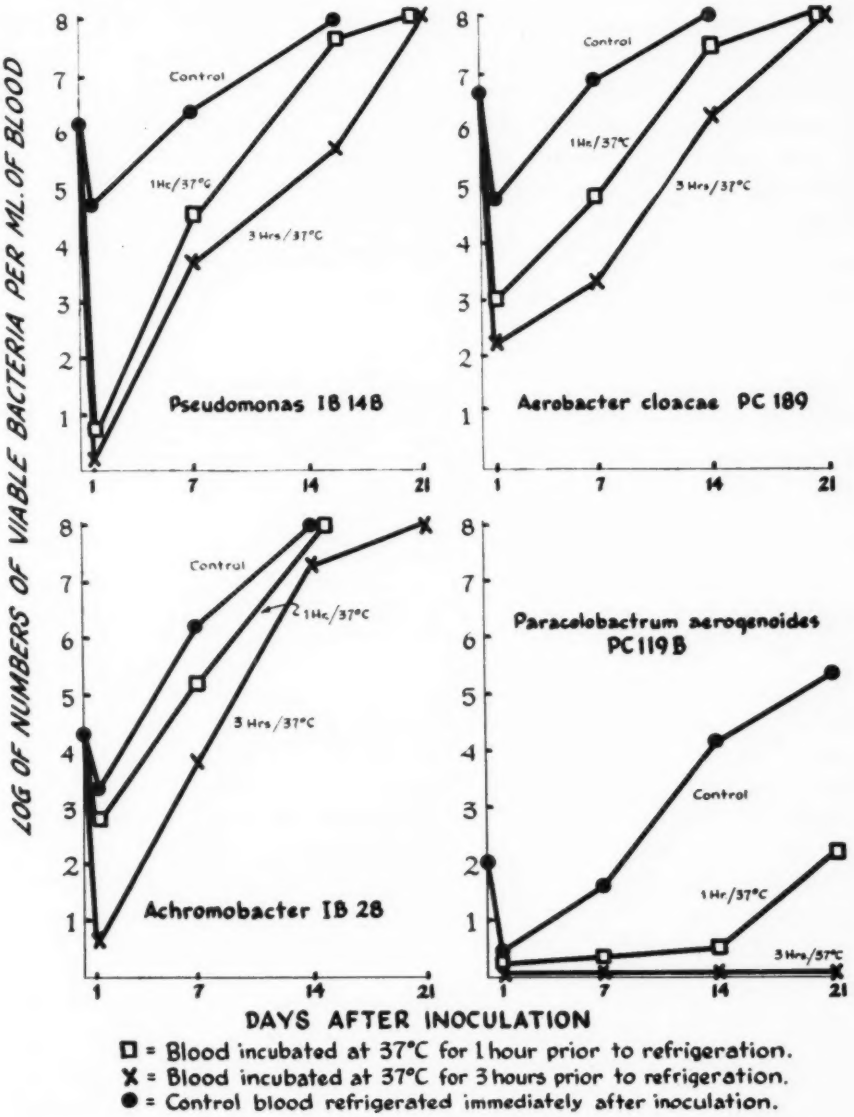
LOG OF NUMBERS OF VIABLE BACTERIA PER ML. OF BLOOD
Pseudomonas 18 148
Aerobacter cloacae PC 189
Achromobacter IB 28
Paracolobactrum aerogenoides PC119B
Control
1 Hr/37°C
3 Hrs/37°C
DAYS AFTER INOCULATION
□ = Blood incubated at 37°C for 1 hour prior to refrigeration.
X = Blood incubated at 37°C for 3 hours prior to refrigeration.
● = Control blood refrigerated immediately after inoculation.

Fig. 1.—Persistence of cold-growing bacteria in bank blood.

fourteenth day, most of the incubated bloods contained 10^7 bacteria or more per milliliter. When small numbers of bacteria (100 organisms per milliliter) were inoculated, a somewhat different sequence of events was observed. Growth in the control tubes usually began within a few days after inoculation and high concentrations (10^8 or more cells per milliliter) were usually present at the

end of 21 days. On the other hand, growth in the incubated blood samples was frequently delayed for at least 7 days and sometimes longer. A greatly extended lag is known to occur when small numbers of cold-growing bacteria are inoculated into refrigerated blood.[19, 20] In our experiments the lag was even more prolonged when the blood was held at a temperature of 37° C. for 1 to 3 hours prior to refrigeration. This delay in the initiation of bacterial growth in the incubated bloods was also reflected in the smaller number of organisms present at the end of 21 days as compared to the controls. Nevertheless, it must be re-emphasized that small inocula of cold-growing bacteria were usually able to multiply and reach high enough levels in the refrigerated blood to constitute a potentially grave threat to the recipient if such blood was transfused. This was true in spite of some initial bactericidal or bacteriostatic activity of the blood against the particular organism.

DISCUSSION

The bactericidal or bacteriostatic properties of whole fresh blood are presumed to be due to the presence of antibodies and complement,[19] properdin,[22] and possibly to viable phagocytic white blood cells.[7] Fortunately, the vast majority of bacterial contaminants which gain access to bank blood during collection are quickly eliminated by these natural factors and the low storage temperature. In some cases, however, the contaminants may persist and, if they are capable of multiplication at low temperatures, may reach concentrations sufficiently high to cause a serious or fatal reaction when the blood is transfused.

We have demonstrated in this study that the natural antibacterial properties of fresh whole blood may be considerably enhanced by incubation at 37° C. for brief periods prior to storage of the blood at 4° C. Such incubation was considerably more effective in sterilizing the blood against those bacteria which grew best at 37° C. than it was against those organisms which grew more rapidly or exclusively at lower temperatures. This apparently anomalous finding may be explained by a combination of two effects: the activity of specific antibacterial blood factors, and the influence of low temperatures on bacterial growth. It is well recognized that the bactericidal action of antibodies and complement follows an exponential curve and occurs more rapidly at 37° C. than at lower temperatures.[23] Very low temperatures may, in fact, inhibit the killing of the organisms by preventing the absorption of complement to the bacteria-antibody complex,[24] inhibiting the action of properdin[22] and inactivating phagocytic white cells.[7] With the warm-growing bacteria, the brief incubation of blood at 37° C. probably accelerated the rate of killing by the antibacterial factors present and the survivors, unable to multiply at 4° C., eventually died out. These same factors may have been equally active at 37° C. against the cold-growing bacteria, but the survivors in this instance were not suppressed by low temperatures and in time multiplied to high levels. Thus, preliminary incubation of whole blood prior to refrigeration is not a reliable or effective method for reducing the incidence of bank blood contamination.

The demonstration that relatively small numbers of many strains of cold-growing Pseudomonas, Achromobacter, Paracolobactrum, Aerobacter, and Escherichia were able to multiply rapidly enough in refrigerated blood to reach potentially dangerous concentrations clearly indicates that constant storage of bank blood at 4° C. is not in itself an adequate means of inhibiting bacterial growth or of insuring sterility of the blood. This is in accordance with reports that these same types of cold-growing organisms are most often implicated in serious or fatal transfusion reactions.[2, 19, 21] Since it seems almost impossible to prevent a few bacteria from getting into bank blood occasionally, it appears inevitable that additional contaminated blood reactions will occur until some effective means, perhaps the addition of antimicrobial substances to blood, are found to suppress the growth of all types of contaminants.

SUMMARY

The normal antibacterial properties of whole fresh blood for many types of gram-negative bacteria could be significantly enhanced by preliminary incubation at 37° C. for brief periods prior to storage of the blood in the refrigerator. Such preincubation was more effective in sterilizing the blood of strictly warm-growing bacteria than it was for those organisms capable of growth at low temperatures. With small inocula of cold-growing bacteria, growth in the incubated bloods was frequently delayed for 7 days or more, but by the end of 21 days, large numbers of organisms were almost invariably present. Any given blood suppressed the growth of different strains of bacteria to a different degree and no dependence could be placed on any blood to eliminate a particular chance contaminant. Storage of bank blood at refrigerator temperature is not in itself an adequate means of inhibiting all types of bacterial growth or of insuring sterility of the blood.

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RADIOACTIVE VITAMIN B_{12} STUDIES

Experience With the Urinary Excretion Test and the Measurement of Absorbed Plasma Radioactivity

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RADIOACTIVE vitamin B_{12} has become a valuable tool in the study of absorption and deposition of vitamin B_{12} in normal individuals and patients with megaloblastic anemias. The reports on the use of cobalt^{60}-labeled vitamin B_{12} in the diagnosis of megaloblastic anemias thus far have utilized indirect methods to study the absorption of the orally administered vitamin. These methods are (a) the study of fecal radioactivity, in which the unabsorbed fraction is measured,[1] (b) the hepatic uptake or deposition of the vitamin B_{12} after oral administration,[2] and (c) the measurement of urinary radioactivity following oral administration when a "flushing dose" of nonradioactive vitamin B_{12} is given.[3] Two of these methods require the patients' cooperation in the collection of samples. In addition, the urinary excretion method has certain limitations reported below.

Preliminary data on the direct measurement in plasma of absorbed vitamin B_{12} have recently been reported, using cobalt^{56}-labeled[4]† and cobalt^{60}-labeled[5] vitamin B_{12}.

It is the purpose of this report to give our experience with (1) the urinary excretion method and (2) the results of the direct plasma measurement of the absorbed radioactive vitamin B_{12}.

MATERIAL

The study includes 38 hematologically normal individuals and nine patients with pernicious anemia. The diagnosis of pernicious anemia was established on clinical history, peripheral blood smear, and bone marrow studies.

The urinary excretion test, using cobalt^{60}-labeled vitamin B_{12}, was performed on 19 normal individuals and on seven patients with pernicious anemia. Plasma radioactivity studies, using both cobalt^{60}- and ^{58}-labeled vitamin B_{12}, were done in 16 normal individuals and five patients with pernicious anemia. Four of the latter patients also had urinary excretion tests.

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†This report has been published recently in more complete form by Booth, C. C., and Mollin, D. L.: Plasma, Tissue and Urinary Radioactivity after Oral Administration of ^{56}Co-labelled Vitamin B_{12}, Brit. J. Haemat. 2: 223, 1956.

METHODS

The cobalt^{60}-labeled vitamin B_{12} employed had a specific activity of 1,082 μc per milligram, while the cobalt^{58} material* contained 611 μc per milligram when originally obtained.

1. *Urinary Excretion.*—The method is essentially that of Schilling.[3] Cobalt^{60}-labeled vitamin B_{12}, 0.46 μg, having an activity of 0.49 μc, was given to the patient in the fasting state. The "flushing dose" of 1,000 μg of nonradioactive vitamin B_{12} was given intramuscularly one hour thereafter. Breakfast was given at the same time. Twenty-four-hour urine samples were collected. A five-hundred milliliter sample was counted on top of a scintillation well counter for 15 minutes. The standard containing 0.49 μc of cobalt^{60}-labeled vitamin B_{12} in 500 ml. of water was counted in the same way. Correcting the net count of the 500 ml. sample for the 24-hour urine volume and dividing this by the net standard counts gave the percentage of the oral dose excreted in 24 hours. Counting errors with this method were less than 5 per cent in pernicious anemia patients with 1 per cent excretion.

2. *Plasma Radioactivity Studies.*—Initial studies with 0.49 μc of cobalt^{60}-labeled vitamin B_{12} in three normal individuals, given in the fasting state without a "flushing" injection, showed no detectable radioactivity in 5 to 7 ml. of plasma at 2, 5, 7, 10, 14 hours after oral administration. With use of 1 μc or more of either cobalt^{60}- or cobalt^{58}-labeled vitamin B_{12}, significant radioactivity was noted as early as 4 hours. Seven milliliters of plasma was counted in a scintillation well counter,† for 30 minutes in the normal individual. In patients with pernicious anemia, in whom we suspected low counts, samples were counted for 45 minutes. The results are given as per cent of the administered dose per liter of plasma. Various experimental procedures, counting method and statistics, and calculations are given in the footnote.‡

RESULTS AND COMMENT

a. *Urinary Excretion Test.*—The results in 26 patients with the urinary excretion method are shown in Table I. In hematologically normal individuals without renal disease the percentage of the dose excreted in 24 hours ranged from 6.2 to 33.4 per cent, with a mean of 17.9 per cent, whereas the pernicious anemia patients had results ranging from 0.20 to 3.74 per cent. Thus there was no overlap in the results between these groups. In five patients with renal disease, the urinary excretion was essentially within normal range in three, but two patients gave false positive results for pernicious anemia. The

*Kindly supplied by Dr. N. S. Ritter, Merck & Company, Rahway, N. J.

†Nancy Wood Counter Laboratories, Chicago, Ill.

‡Diluted samples prepared from the radioactive vitamin B_{12} stock solution were counted before and after the addition of various volumes of water. Up to 7 ml. the efficiency of our well counter for cobalt^{60} or cobalt^{58} did not change. This volume was therefore selected for future counting. With a 10 ml. volume there was a 50 per cent loss in efficiency as compared to 7 ml. for the same total radioactivity. An attempt was made to precipitate the plasma proteins with trichloroacetic acid, hoping to concentrate the bound vitamin B_{12} in larger than 7 ml. of plasma, but as much radioactivity was found in the supernatant as in the precipitate. Counting statistics: With a background of 4,000 counts for 30 minutes and a net sample count of 500, the probable error in counting was 18 per cent. At 1,000 counts above background, the probable error was reduced to 7 per cent. At 10 hours in the normal patient, the counts were such that there was an error of approximately 10 per cent. Estimation of efficiency: From the known efficiency of a cobalt^{60} standard, daily efficiencies for this isotope were calculated. The efficiency for cobalt^{58} was arrived at from the efficiency for I^{131} and cobalt^{60} for that day.[6] Calculations: The net counts for 7 ml. of plasma counted for 30 minutes were converted to per cent of dose per liter of plasma as shown:

$$1.\ \text{per cent dose/L} = \frac{\text{net counts for 7 ml.} \times f\ (\mu c \times 10^{-2}/L)}{\text{Dose in } \mu c}$$

$$2.\ f\ (\mu c \times 10^{-2}/L) = \frac{\text{Dilution (142.8)}}{\text{0.efficiency} \times 2.2 \times 10^{6}\ (\text{dis./min./}\mu c) \times 30 \text{ minutes}}$$

$$= \frac{0.216 \times 10^{-8}}{\text{0.efficiency for cobalt}^{60} \text{ or cobalt}^{58}}$$

Since cobalt^{60} gives off two gamma rays its counting efficiency is twice that of cobalt^{58}.

TABLE I. URINARY EXCRETION OF COBALT^{60}-LABELED VITAMIN B_{12} IN PERNICIOUS ANEMIA PATIENTS, AND HEMATOLOGICALLY NORMAL INDIVIDUALS WITH AND WITHOUT RENAL DISEASE (USING 0.46 μG OF THE VITAMIN)

NO.	AGE SEX	DIAGNOSIS	SERUM UREA NITROGEN (MG. %)	SERUM CREAT. (MG. %)	CRE-ATININE CLEARANCE (ML./MIN.)	URINE VOLUMES (ML.)	% B_{12} EXCRETED (% OF DOSE)
1	50-F	Pernicious anemia, exacerbation	18.5			1,635	2.36
2	79-F	Pernicious anemia, exacerbation	25.0			625	0.20
3	87-F	Pernicious anemia, remission	20.0	0.7		350	0.28
4	43-M	Pernicious anemia, remission	30.0			1,035	3.74
5	77-M	Pernicious anemia, remission				1,090	1.05
6	87-F	Pernicious anemia, remission	27.0	0.9		1,935	2.05
7	68-M	Pernicious anemia, remission				625	0.22
8	58-M	Chronic glomerulonephritis, nephrotic stage	19.5	1.0	53.0	1,450	25.20
9	50-M	Chronic glomerulonephritis	74.0	4.3	17.0	1,120	6.0
10	35-F	Diabetes mellitus, chronic pyelonephritis	81.0	7.6	8.6	1,990	17.2
11*	52-M	Hypertensive cardiorenal disease, terminal	110.0	9.7		250	0.1
12*	54-M	Hypertensive cardiorenal disease, terminal	134.0	18.9		240	0.3
13-26†		Hematologically normal without renal disease	Range			760-2,740	6.2-33.4
			Mean				17.9

*These two patients were receiving intravenous fluids and had an indwelling catheter to insure complete 24-hour urine collections.

†Includes two patients with subtotal gastrectomies.

urine volumes of these two patients were low; however, they were accurate 24-hour specimens since the collection was made by catheter. It appears that chronic renal disease may affect the excretion of vitamin B_{12}. Rath and coworkers[7] noted a similar effect of chronic renal disease in seven of eight patients who excreted less than 2 per cent of the administered dose. Intrinsic factor preparations did not alter the results.[7]

b. *Plasma Radioactivity With 2 μg or Less of Vitamin B_{12}.*—The plasma radioactivity studies with 2 μg or less of cobalt^{60}-, or cobalt^{58}- labeled vitamin B_{12} are shown in Table II and Fig. 1. The peak value of radioactivity was attained at 10 hours after the oral administration of the radioactive tracer dose and it then declined, still showing significant levels at 24 hours. The range of levels at 10 hours in the normal individual was from 0.23 to 1.71 per cent of dose per liter of plasma, with a mean of 0.8 per cent. In patients with pernicious anemia who were in remission, the plasma radioactivity never rose above 0.12 per cent at any time. Previous studies[4] have shown the peak values in normals to occur between eight and twelve hours, but minimal[5] or no detectable[4] radioctivity was found in patients with pernicious anemia. Following potent intrinsic factor preparations the pernicious anemia patients showed similar absorption curves to the normal.[4] The clearance of radioactivity from

TABLE II. PLASMA RADIOACTIVITY AFTER ORAL ADMINISTRATION OF COBALT^{60}- AND ^{58}-LABELED VITAMIN B_{12} IN HEMATOLOGICALLY NORMAL AND PERNICIOUS ANEMIA PATIENTS (USING LESS THAN 2 μG OF THE VITAMIN)

NO.	AGE SEX	DIAGNOSIS	PLASMA RADIOACTIVITY AT HOURS (% DOSE/LITER)										ISOTOPE	DOSE (μC)	DOSE (μG)	% B_{12} EXCRETED
			4	5	6	7	8	9	10	12	14	24				
1	31, F	Eosinophilia		0.72		1.34			1.32		1.27		60	1.49	1.38	
2	37, F	Bronchial asthma			0.59			0.66		0.56			60	1.49	1.38	
3	25, F	None	0.22			0.41			0.51				60	1.49	1.38	
4	53, M	Hemangioma of spinal cord		0.0			0.22		0.23		0.27	0.16	58	1.06	2.0	
5	63, M	Atrophic gastritis		0.027			0.35		0.41		0.38	0.25	58	1.06	2.0	
6	58, M	Arteriosclerotic heart disease		0.0			0.44		0.34		0.27	0.34	58	1.06	2.0	
7	31, F	Multiple sclerosis		0.36			0.36		0.55		0.64	0.29	58	1.06	2.0	
8	50, M	Myocardial infarct		0.11			0.52		0.68		0.58	0.47	58	1.06	2.0	
9	77, F	Malignant melanoma		0.65			0.63				0.48	0.42	58	1.06	2.0	
10	35, F	Adrenogenital syndrome		0.51			0.64		0.67		0.58	0.39	58	1.06	2.0	
11	44, M	Epilepsy		0.20			0.93		1.10		0.84	0.58	58	1.06	2.0	
12	25, F	Diabetes mellitus		0.54					1.28		1.05	0.69	58	1.06	2.0	
13	30, F	Rheumatic heart disease		0.98			1.66		1.71		1.59	1.00	58	1.06	2.0	
		Mean		0.46			0.64		0.80		0.72	0.46				
		Range		0.0-0.98			0.22-1.66		0.23-1.71		0.27-1.59	1.00-0.46				
14	77, M	Pernicious anemia*		0.11				0.12		0.05			60	1.49	1.38	1.05
15	87, F	Pernicious anemia*		0.0			0.11						60	1.49	1.38	2.05
16	68, M	Pernicious anemia*							0.022				60	1.49	1.38	0.22
17	56, F	Pernicious anemia*							0.080				60	1.49	1.38	

*In remission.

the plasma was not affected by either remission or relapse.[4] In both of these preliminary reports[4, 5] the data are given in counts per minute, so that we are unable to compare their data with ours.

c. *Plasma Radioactivity With 4 μg of Vitamin B_{12}.*—Plasma radioactivity studies were performed on three normal patients and two with pernicious anemia, with use of 4 μg of vitamin B_{12} (Table III). In three normals the maximal plasma levels were about half of the mean value of 0.8 per cent found at 10

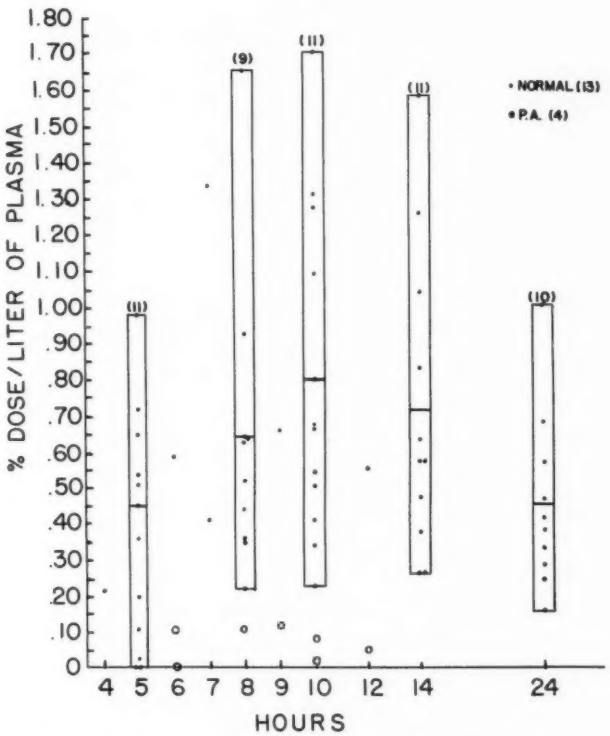

Fig. 1.—Plasma radioactivity after oral administration of cobalt-labeled vitamin B_{12} (less than 2 μg of vitamin).

TABLE III. PLASMA RADIOACTIVITY AFTER ORAL ADMINISTRATION OF 4 μG OF COBALT^{58}-LABELED VITAMIN B_{12}

NO.	AGE SEX	DIAGNOSIS	PLASMA RADIOACTIVITY AT HOURS (% DOSE/LITER) 8	10	14	24	DOSE (μC)	% B_{12} EXCRETED
18	M	Arteriosclerotic heart disease	0.42	0.46	0.31	0.25	1.97	
19	64-F	Cerebrovascular accident	0.56	0.46	0.40	0.43	1.97	
20	72-M	Cerebral embolus	0.45	0.42	0.32	0.32	1.97	
21	87-F	Pernicious anemia, remission	0.11	0.032			1.84	0.28
15*	87-F	Pernicious anemia, remission	0.42	0.32			1.84	2.05

*Repeat study on patient previously tested with 1.38 μg.

hours when 2 μg or less of vitamin B_{12} was used. This seems to bear out previously reported data that absorption of vitamin B_{12} in the gastrointestinal tract of normal individuals is decreased if the dosage is above 1.5 to 2.0 μg.[8] In Patient 15 with pernicious anemia, who was previously studied with the urinary excretion test and the plasma radioactivity study using 1.38 μg of cobalt^{60}-labeled vitamin B_{12}, the plasma radioactivity levels after 4 μg were within the normal range. This seems to imply that with larger amounts of vitamin B_{12} there may be a process other than active absorption, possibly simple diffusion. This needs further investigation.

It is evident that the ability to pick up radioactivity in the plasma depends on the amount of activity of radioactive vitamin B_{12} administered to the patient. Since the maximum permissible body content for cobalt^{60} is 3 μc, this isotope could not be used for repeat studies. At least 1 μc is necessary to give significant plasma radioactivity by our method of counting. The use of shorter half-life isotopes of cobalt, either ^{58} or ^{56}, with high specific activities, will solve this problem. The cobalt^{56}-labeled vitamin B_{12}, used by Booth and associates,[4] had a specific activity of 5,000 μc per milligram and enabled them to give 1 μg of vitamin B_{12} having an activity of 5 μc. Counting time and sample size could be reduced with this. Unfortunately, vitamin B_{12} tagged with one of the shorter half-life cobalt isotopes, having a high specific activity, is as yet not commercially available in the United States.

The low plasma levels as compared to higher absorption figures with the urinary and fecal excretion studies bear comment.

If plasma volume makes up about 3.8 per cent of body weight, then in a 60 kilogram man the plasma volume would be 2.25 L. At 10 hours after the administration of tagged vitamin B_{12} the mean plasma level was found to be 0.8 per cent of the dose per liter. Assuming distribution of the absorbed vitamin into plasma volume only, about 2 per cent of dose would be absorbed. Since fecal and urinary studies indicate absorption to be much higher in the normal, the distribution of the absorbed vitamin B_{12} must be greater than plasma volume.

SUMMARY

The results with the cobalt^{60}-labeled vitamin B_{12} urinary excretion test on 19 normal individuals and seven pernicious anemia patients, using 0.46 μg of vitamin B_{12}, have been presented. In the normal patients without renal disease the range was 6.2 to 33.4 per cent of administered dose excreted in 24 hours. Patients with pernicious anemia excreted less than 3.7 per cent of the dose. Severe renal disease with oliguria was found to produce false positive excretion studies. Plasma radioactivity as a more direct measure of absorbed vitamin B_{12} was found to be a feasible method for diagnostic use if 1 μc or more of tagged vitamin B_{12} was used. The peak plasma activity occurred at 10 hours after the oral administration of the tracer dose. In 13 normal patients receiving 2 μg or less of vitamin B_{12} the plasma activities ranged from 0.23 to 1.71 per cent of dose per liter of plasma, with a mean of 0.8 per cent,

while in four patients with pernicious anemia the levels never rose above 0.12 per cent. It is felt that the plasma radioactivity method has much to offer since it does not require the patient's cooperation in the collection of specimen.

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THE INTERRELATIONS OF BLOOD CALCIUM AND CITRIC ACID

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HYPERCITRICEMIA has been reported in association with elevated blood calcium levels in hyperparathyroidism,[1-5] vitamin D intoxication,[5-9] certain cases of osteolytic metastases to bone,[5, 10] and under circumstances of prolonged immobilization.[5] The converse of this situation, hypocalcemia with low blood citric acid levels, has been noted in hypoparathyroidism,[1, 3, 5, 8, 11-13] in newborn infants,[14] and occasionally in vitamin D-deficient rickets.[7] This apparent relationship has stimulated vigorous speculation concerning the influence of calcium concentration on the observed changes in citric acid. Many investigators have implied and some have stated that the change in calcium concentration are responsible for the reported variations in blood citrate levels in these circumstances.[2-4, 15, 16]

In the course of a continuing study of calcium infusions the effect of acute hypercalcemia on blood citric acid levels was studied in normal man.

METHODS

All subjects were young, ambulatory, hospitalized patients or laboratory personnel without renal, hepatic, or skeletal disease. They were studied in the postabsorptive state while resting quietly in bed. All infusions were 500 ml. in volume and were administered over a 3-hour period by gravity drip. The infusions were made up to contain 10 mg. per kilogram of calcium as calcium gluconate†; however, no less than 600 mg. of calcium was infused in any subject. The calcium was infused in isotonic saline and in 5 per cent dextrose in water. In some subjects, infusions of saline or 5 per cent dextrose in water were given over the same time interval and at the same rate. Blood specimens were obtained prior to, at the end of, and one hour following each infusion.

Each blood sample was analyzed in duplicate for calcium by the technique of Clark and Collip[17] and for citric acid by the method of Hess and White[18] modified in the following manner. Color development was carried out in an Evelyn Macro cuvette. The cuvette was then centrifuged for 10 minutes at 2,000 r.p.m. and transmittance measured in an Evelyn photometer with a 500 mμ filter and the reagent blank set at 100 per cent. The 6 ml. slit was utilized to place the pink layer in the light path.

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RESULTS

Table I summarizes the normal values for citric acid in this laboratory, the reproducibility of the method, and the minimum change in citric acid considered significant. The last value combines the maximum variation in citric acid in noninfused subjects over the time of the experiment (0.4 mg.) and the reproducibility of the method (0.1 mg.). Consequently, no importance was attached to changes in citric acid which did not exceed 0.5 mg.

TABLE I

a. Normal blood citric acid level 2.0 ± 0.5 mg. per 100 ml.
b. Reproducibility of the method 0.1 mg. per 100 ml.
c. Minimum significant change 0.5 mg. per 100 ml.

a, Mean value of 58 determinations in 50 subjects; *b*, value for mean variation of 10 replicate determinations; *c*, maximum fasting variations in 5 noninfused subjects plus the reproducibility figure.

In all 10 patients infused with calcium in isotonic saline, there occurred significant elevation of citric acid. The range was from 0.9 mg. to 2.6 mg. with a mean of 1.64 mg. (Fig. 1). However, the infusion of saline without calcium resulted in an equally impressive rise in citric acid. In this group of 11 subjects, elevation ranged from 0.6 mg. to 3.7 mg. with a mean rise of 1.83 mg. (Fig. 1).

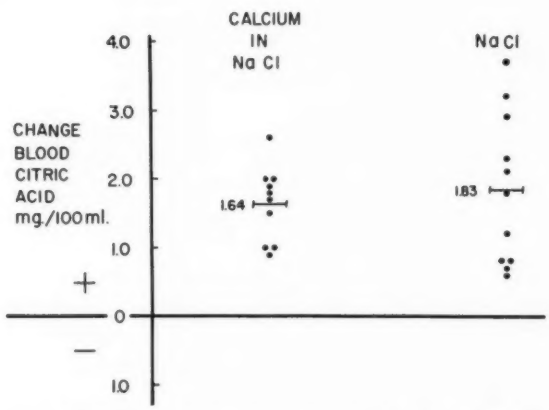

Fig. 1.—Changes in blood citric acid levels after infusions of at least 600 mg. of calcium in saline and equal volumes of saline alone.

When calcium in an amount equal to that infused in saline was given in glucose to 7 subjects, there occurred no significant rise in citric acid. Changes in this group ranged from –0.1 mg. to +0.6 mg. with a mean of +0.23 mg. (Fig. 2). The administration of dextrose in similar fashion to 7 subjects resulted in a mean decrease in citric acid of 0.3 mg. The range was from +0.2 mg. to –0.7 mg. (Fig. 2).

A comparison of the elevations in calcium and the changes in citric acid in the groups infused with calcium and saline and calcium in glucose is presented in Fig. 3. In the calcium-saline group a prominent increase in citric acid (mean 1.64 mg.) was associated with a substantial increment in serum

calcium (mean 2.2 mg.). Although the subjects infused with calcium in glucose demonstrated an even greater rise in calcium (mean 3.01 mg.) there occurred an insignificant change in citric acid (0.23 mg.).

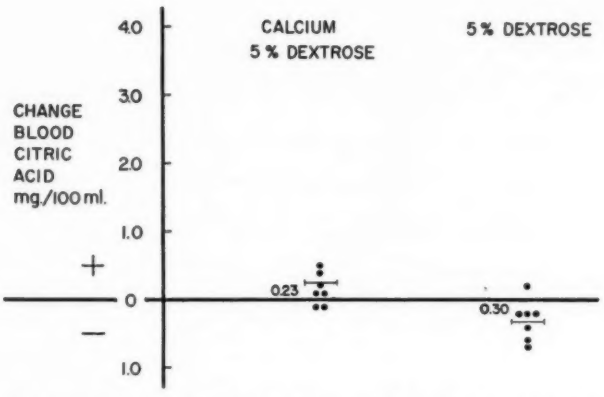

Fig. 2.—Changes in blood citric acid levels after infusions of at least 600 mg. of calcium in 5 per cent glucose and equal volumes of 5 per cent glucose alone.

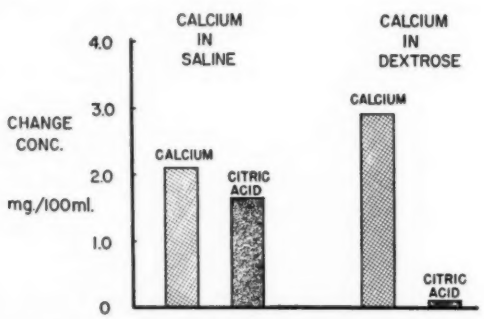

Fig. 3.—Mean changes in calcium and citric acid in 10 subjects infused with at least 600 mg. of calcium in 500 ml. of isotonic saline compared with those in 7 subjects infused with equal amounts of calcium in 500 ml. 5 per cent glucose in water.

COMMENTS

The reported instances of hypo- and hypercitricemia in conditions associated with depression or elevation of blood calcium would appear to support the assumption of a direct association. There have been, however, relatively few studies which serve to illuminate this problem. These have been directed along three principal avenues: (1) the changes induced in animals and man by vitamin D deprivation and the response of blood and tissue calcium and citrate to its administration; (2) the effects of parathyroid deficiency and the changes induced by exogenous parathyroid hormone in normal and hypoparathyroid subjects; (3) the effects of a variety of salt and citric acid infusions on the calcium and citric acid levels of animals.

In animals, Carlsson and Hollunger[19] noted that vitamin D in both physiologic and toxic doses caused a rapid (less than 24-hour) increase in blood citrate without concomitant change in calcium levels.[19] Citrate also rose in bones and teeth. Sudden increase in calcium intake in animals on a low cal-

cium diet resulted in elevation of citrate, but this change did not occur with the rapidity seen following vitamin D treatment and was believed to be the result of a dissimilar physiologic mechanism. Steenbock and Bellin[20] noted in both normal and rachitic rats similar elevation of citrate after vitamin D therapy unrelated to serum calcium or phosphorus levels. Chang and Freeman,[9] using thyroparathyroidectomized dogs, found parallel changes in calcium and citric acid before and after vitamin D, and suggested an influence of this vitamin on the metabolism of both substances. Harrison[5, 7, 8] reported consistently low levels of citrate unrelated to serum calcium concentration in untreated rachitic infants. Following vitamin D therapy a "partial correlation" was suggested by the elevation of both calcium and citric acid levels. However, Ochse[21] found no abnormalities in citrate levels in rachitic infants and noted that a single dose of vitamin D resulted in a drop in citric acid. In adults, Anning and associates[6] reported that toxic doses of calciferol resulted in hypercalcemia and an elevation of the diffusible, nonionized fraction of serum calcium and suggested that this was due to an increase in complexed calcium. In this connection it may be noted that McLean[22] considers the complexed calcium an insignificant fraction of total serum calcium under normal circumstances in man, although in the rabbit it is known that considerable amounts of diffusible nonionized calcium exist as a complex with citric acid.

The instances of hypocalcemia and hypocitricemia in hypoparathyroidism and elevations in blood calcium and citrate in hyperparathyroidism have been duplicated in animal studies. Chang and Freeman[9] demonstrated a simultaneous depression in calcium and citrate after thyroparathyroidectomy in dogs. Similar changes have been found in thyroparathyroidectomized rats by L'Heureaux and Roth.[23] A marked decrease in bone citrogenase activity and hypocalcemia has been found in rats, four to six weeks after thyroparathyroidectomy without alteration in bone citric acid levels.[24] Dickens[16] reported an increase in bone citrate in a puppy treated with parathyroid extract. Parathyroid extract has induced hypercalcemia and hypercitricemia in normal dogs[3] and rats.[23] These data, as well as the discovery of the large quantity of citric acid in normal bone[16] and the demonstration of enzyme systems capable of producing citric acid in quantities which vary directly with the metabolic activity of the area of bone studied[24] and with the presence or absence of parathyroid glands,[25] support the concept of an intimate relationship between calcium and citric acid. They do not, however, indicate direct relationship between their respective concentrations.

The third type of investigative effort has produced conflicting results. Calcium chloride infusions caused no change in blood citrate in dogs,[9] but in the rabbit a marked elevation ensued.[26] Calcium lactate and strontium chloride infusions resulted in marked elevations of citrate in rabbits.[26] Infusions of sodium hydroxide caused no change in rabbits or dogs.[9, 26] Infusions of citric acid were reported to result in an elevation of citrate in dogs by Gomori and Gulyas[27] while Chang and Freeman[28] found no change. These studies may be variously interpreted as favoring a calcium effect on citrate, a citrate effect on calcium, or no effect from either.

Depression in blood citrate has been reported following both oral and intravenous glucose in animals and man.[29-33] The insignificant changes in citrate found in our subjects infused with glucose are best explained by the small quantities of glucose used, i.e., 0.3 Gm. per kilogram over a 3-hour period. In all other studies 1 Gm. or more per kilogram was administered over a much shorter period and blood samples were obtained at more frequent intervals. We have found a significant reduction in citric acid when 1.0 or 1.5 Gm. per kilogram of glucose is infused over a 3-hour period.[34] Since insignificant depressions of citric acid followed the smaller glucose infusions, the lack of elevation in citrate in the subjects infused with calcium and the same amount of glucose appears significant. It should be stated, however, that from these data some glucose effect cannot be fully ruled out.

The elevations in citric acid in the group infused with saline are of greater interest. Others have infused saline in rabbits[26] and dogs[28] without change in citrate. In the study reported by Chang and Freeman one blood specimen was obtained after a period of one hour. When calculated on the basis of milligrams per kilogram per hour of solute infused we gave smaller amounts than those used by Chang and Freeman or Alwall, but the total solute in milliequivalents or millimols infused in the present study was much greater. Therefore the changes in citrate noted here may be due to the quantity of sodium chloride infused or to a temporal factor. Preliminary studies are in progress to determine effects of the infusion of varying quantities of different anions and cations on the blood citrate level and to assay the temporal factors of the response.

The observation of hypercitricemia without hypercalcemia in patients with active Paget's disease by Harrison[8] and by Kissin and Kreeger[35] serves as strong clinical evidence for lack of direct influence of calcium on blood citric acid levels. Such observations, together with the results of this study suggest that the changes in citric acid concentration noted in various skeletal disorders are the result of alterations in citrate homeostasis due to the basic disease process, rather than to the associated changes in serum calcium.

SUMMARY

Study was conducted of the effects of acute hypercalcemia on blood citric acid in groups of normal subjects by infusing calcium gluconate, glucose, and saline in various combinations. Significant elevation of blood citric acid accompanied the infusion of calcium in saline but an equal degree of hypercitricemia resulted from the infusion of normal saline in equal amounts over a similar interval.

No significant changes in blood citrate acid resulted from the infusion of calcium in glucose or of glucose alone. The degree of hypercalcemia attained by infusion of calcium in glucose equaled that induced by calcium in saline, but only in the latter instance did blood citrate rise significantly.

This study suggests that alterations in citrate observed in a variety of skeletal disorders are related to the basic disease process rather than to changes in serum calcium. These data offer no satisfactory explanation for the hypercitricemia which resulted from infusion of isotonic saline.

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EVALUATION OF THE EMBRYONATED HEN'S EGG AS A HOST FOR THE AGENT OF INFECTIOUS HEPATITIS

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IN A recent review assessing the outcome of the numerous efforts to adapt the agent of infectious hepatitis to the embryonated hen's egg, Henle[1] came to the conclusion that the susceptibility of this laboratory host, like that of others,[2] was not proved, in spite of the fact that the infectivity of inoculated eggs has been tested in human volunteers and mild illness resembling anicteric hepatitis has resulted.[3-6] The importance of the disease and the relative scarcity of direct data have prompted us to report our own experiences with forty-nine human volunteers who were fed tissues and fluids from embryonated hens' eggs injected with infectious hepatitis (IH or hepatitis A) inocula. Three of the first ten subjects developed mild atypical illnesses after swallowing yolk sac inoculum, but attempts to verify this result by repetition were unsuccessful. Under the conditions of the experiments, the embryonated hen's egg appeared to have deficiencies as a host for study of infectious hepatitis.

MATERIALS AND METHODS

Sources of Inoculum.—Two IH strains were studied. One came from J. F., a patient in a grumbling outbreak at the Rome (N. Y.) State School, that had been going on for almost two years. There were over 60 cases with no deaths, critical illnesses, or serious complications during the 6-month period in which J. F.'s illness occurred. Consequently, it was felt that if IH strains vary in virulence, one from this outbreak would be relatively mild, a highly desirable property in an agent to be tested in humans. J. F. ran a typical clinical course of mild hepatitis with jaundice at a time when the epidemic curve was at its peak and there were many other cases occurring. Hence, the diagnosis of IH was established as firmly as is possible in the present state of our knowledge. Specimens of serum obtained† three days after onset constituted the inoculum. Aliquots were distributed into tubes, quick frozen in a dry-ice alcohol mixture, and stored at temperatures ranging between −20° and −70° C.

The Akiba strain was kindly furnished by Drs. Werner Henle and Miles Drake of the Children's Hospital of Philadelphia. It originally came from an outbreak of IH in a

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†Through the kindness of Dr. Robert F. Korns.

summer camp studied by Neefe and Stokes[7] and had been maintained by direct passage in volunteers. Specimens used to inoculate embryonated hens' eggs included both serum and stool specimens collected during acute illness from several different volunteers studied by the Philadelphia group and in our own series. Details are given later.

Inoculation and Harvesting of Embryonated Hens' Eggs.—Passages were carried by the amniotic or yolk sac routes. Fecal or tissue suspensions were made in broth according to wet weight; serum or amniotic fluids were inoculated undiluted. Amniotic inoculations were done with 0.1 ml. volumes in 6- or 7-day-old eggs. After 7 to 10 days of incubation at 35 to 36° C., amniotic fluid and embryo body were harvested and used for passage. Embryo was usually ground in amniotic fluid plus broth to make a 20 per cent suspension. For yolk sac passages, 5- or 6-day eggs were inoculated with 20 per cent yolk sac and in some cases 50 per cent embryo body suspensions, totaling 0.1 to 0.5 ml. in volume, and were harvested after 7 to 10 days of incubation. Between passages tissues or fluids were sometimes stored in the CO_2 box.

Volunteers.—Healthy, psychologically stable men between 21 and 30 years old were volunteers. History, physical examination, hematologic examination including sedimentation rates, urinalyses, repeated fecal examinations for enteric pathogens, studies of pharyngeal flora, chest x-rays, and liver function tests revealed no abnormalities in volunteers accepted for the study. Subjects underwent a preliminary period of isolation and observation following acceptance, during which frequent physical examinations, liver function tests, and other laboratory examinations were done.

Quarters, Staff, and Clinical Procedures.—The hospital quarters consisted of two cell blocks in the New York State Vocational Institution, West Coxsackie, New York, which were separated by a common room adjacent to which there were kitchen facilities. Each cell contained a wash basin and toilet and could be closed off from the corridor by a door. Stringent precautions for isolation were enforced with respect to food, wastes, and contact. Food containers were dry-heat sterilized after each use. Syringes, instruments, or wastes were autoclaved or incinerated. During periods when there was no illness, volunteers in a given experiment were permitted contact with one another, but each group was rigidly segregated from other volunteers. Subjects who developed even trivial symptoms or signs were individually isolated. Volunteers were under the continuous supervision of a registered nurse and an officer of the institution, and were visited once or twice daily by physicians, one of whom was always on call. Only the staff conducting the experiment, who were regularly injected with gamma globulin, were permitted in the hospital quarters, so that opportunities for accidental acquisition of the disease by the volunteers from outside sources were minimum. Comparisons of symptoms and signs were made by a standard check list, temperatures were taken twice or four times daily, and volunteers were weighed daily. Hematologic and other clinical-pathologic examinations were carried out regularly.

Inocula were mixed with milk immediately before they were fed to volunteers.

Liver Function Tests.—Liver function tests were done with fresh specimens by techniques giving the following normal values: Bromsulfalein retention tests, <5 per cent retention 45 minutes after injection of 5 mg. per kilogram[8a]; 1-minute direct serum bilirubin, <0.21 mg. per 100 ml.[8b]; total serum bilirubin, 1.0 mg. per 100 ml.[8b]; thymol turbidity, 5.0 units[8c, 9]; cephalin cholesterol flocculation, $<++$ in 24 hours[10]; thymol flocculation, $<+$ in 24 hours[11]; urinary urobilinogen,[12] <1.9 Ehrlich units[13]; and urinary bilirubin, no visible reaction.[14] Serum tests were performed usually one or more times a week, urine tests daily. Other examinations were done if there were specific indications.

RESULTS

Six trials of the infectivity of embryonated hens' egg passage materials were carried out, three each with lines initiated from JF and Akiba inocula, respectively.

Studies with JF Inocula.—

First experiment: The yolk sac route of inoculation was chosen for the first series of egg passages because this technique ensures wide distribution of inocula, can be performed atraumatically in young embryos, and, in the case of many viruses, is the inoculation route yielding greatest morbidity and mortality. On Nov. 8, 1951, each of 10 volunteers was fed 4 ml. of 20 per cent (by weight) suspension of yolk sac from the fifteenth serial yolk sac passage of the JF strain. Three men responded by developing mild atypical illnesses, on the seventh, tenth, and thirtieth days after inoculation, respectively (Figs. 1-3).

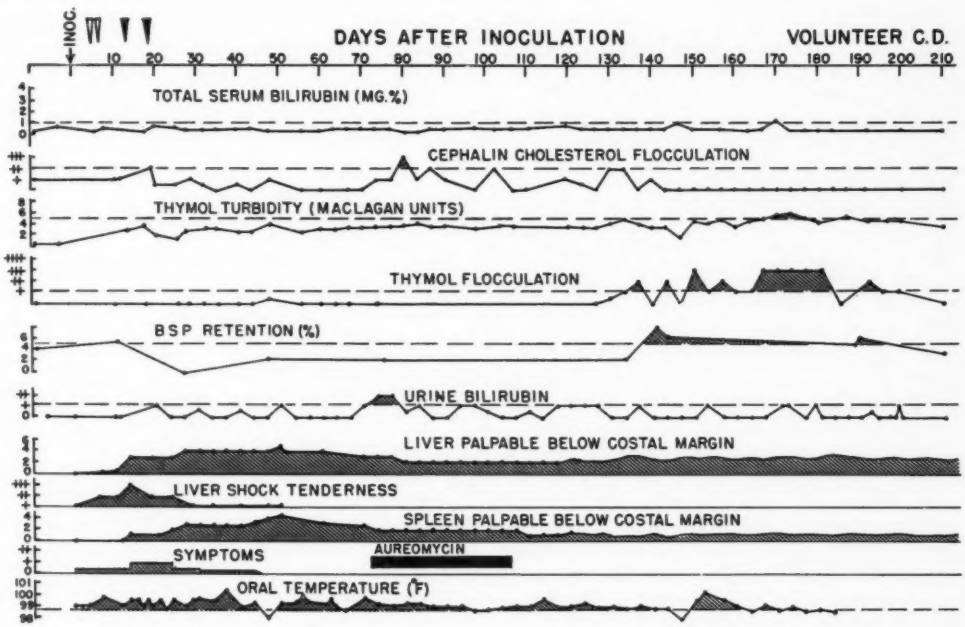

Fig. 1.—Clinical chart of Volunteer C. D. of the first experiment. On the days indicated by pips at the top of the figure, serum was obtained and stored for subinoculation into other volunteers. That taken at onset of illness, on days indicated by open pips, was pooled with specimens taken at onset from Volunteers W. H. and H. D. That taken after the pattern of illness was better established is signaled by the solid pips. It was similarly used in a second pool. The dotted lines indicate approximate normal value for the liver function tests.

There was no clinical jaundice in these patients nor were tests of liver function ever consistently abnormal. The illnesses were characterized by anorexia, fatigability, abdominal and flank pain and tenderness, enlarged tender livers, and, in two cases, splenomegaly. At times the men exhibited low fever. Recovery was uneventful but took several months. It is possible that these subjects were suffering from an adventitious disease such as infectious mononucleosis, but repeated attempts to establish this or other diagnoses failed.

These equivocal results were encouraging and an attempt to transmit the disease from the ill subjects was planned. Events of the next few weeks illustrated one of the pitfalls of volunteer experiments. A group of 10 subjects

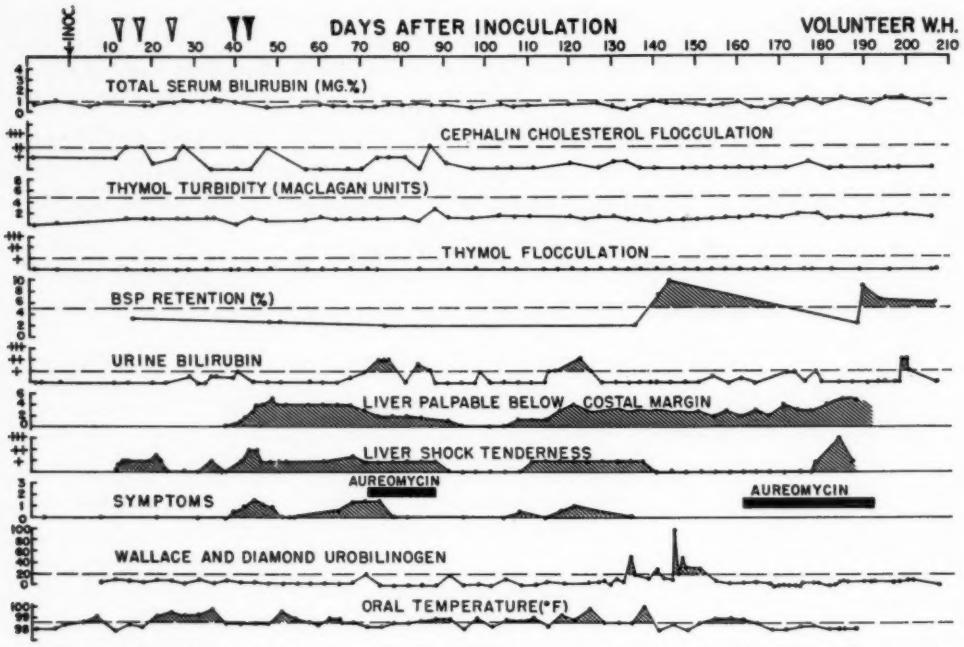

Fig. 2.—Clinical chart of Volunteer W. H. of the first experiment. See Fig. 1 for explanation of symbols.

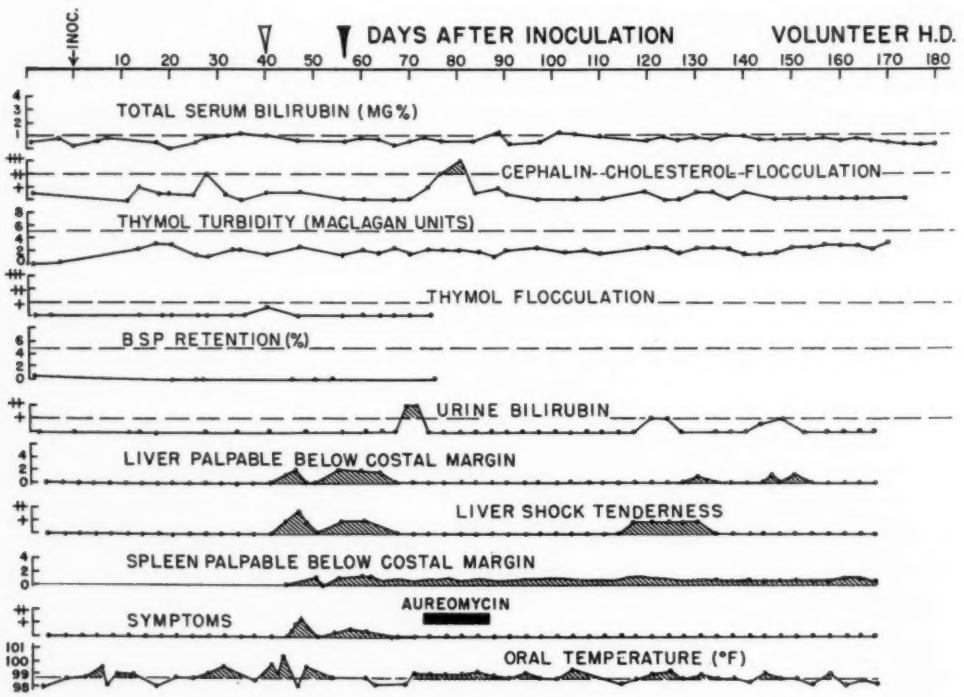

Fig. 3.—Clinical chart of Volunteer H. D. of the first experiment. See Fig. 1 for explanation of symbols.

was recruited and observed in group isolation. They had been screened carefully by the methods mentioned above. In spite of this, one of the new volunteers developed typical hepatitis with jaundice during the preliminary observation period. He had repeatedly denied contact with sources of hepatitis, but later admitted giving himself narcotic injections prior to his entry into State institutions. He had used a needle also employed without sterilization by a number of other persons. The presumptive incubation period was compatible with the diagnosis of serum hepatitis (hepatitis B). The 9 other volunteers in this group were never inoculated but were observed as if they had been and served as control subjects. They remained well. Steps were taken to avoid repetition of this experience, although it is difficult to guard against deliberate deception.

Ten more men were then screened and observed for the attempt to transmit the disease from the 3 mildly ill volunteers. Sera taken from each of the 3 volunteers at onset of symptoms were mixed in a pool, and other sera taken from the same men at the height of clinical illness (Figs. 1-3) were made into a second pool. On Feb. 27, 1952, each of the 10 new subjects was fed 1 ml. of the first pool. The second pool was administered a day later because of the possibility that antibody might have been present in one or more of its component sera; the dose for this inoculation was the same. The volunteers remained well.

Second experiment: Seven-day embryos were injected amniotically with 0.1 ml. of the JF serum and amniotic transfers were carried out with 0.1 ml. volumes of amniotic fluid, sometimes containing embryo brei. On May 6, 1952, each of 8 volunteers was fed a mixture of amniotic fluid (2 ml.) and 50 per cent embryo suspension (5 ml.) from the thirtieth amniotic passage. None of the men developed symptoms, signs, or laboratory evidence of hepatitis.

Third experiment: This was an effort to confirm and extend the results of the first experiment. An aliquot of the thirteenth JF yolk sac passage had been stored at –50° C. It was transferred three more times at ten-day intervals via the yolk sac to offset possible loss of infectivity on storage; then 4 ml. of yolk sac plus 4 ml. of 50 per cent embryo body suspension were fed to seven volunteers on Sept. 16, 1952. All remained well.

Studies With Akiba Inocula.—A pool of sera from 8 volunteers with infectious hepatitis induced with the Akiba strain had kindly been furnished by Drs. Werner Henle and Miles Drake of the Children's Hospital of Philadelphia. The pool had been distributed into aliquots and stored in the frozen state at –50° C. Its infectivity was demonstrated by titration in 13 volunteers. Four of the 13 subjects had already taken part in the second experiment, and on July 17, 1952, each of the 4 was fed 2 ml. of a 1:100 dilution of the pool. Two (Volunteers J. Y. and T. R.) developed typical hepatitis with jaundice after incubation periods of 33 and 37 days, respectively. On Aug. 11, 1952, each of the other 9 volunteers swallowed 2 ml. of dilutions of another aliquot of the pool; 5 were fed 1:1,000 dilution, and 4 were given 1:10,000 dilution. One of

the men inoculated with the 1:1,000 dilution, Volunteer H. E., developed typical hepatitis with jaundice after an incubation period of 35 days. The other 8 subjects remained well.

Undiluted aliquots of the Akiba pool that had not previously been thawed, and also specimens collected during early acute illness for Volunteers J. Y. and H. E., were starting inocula for the further passages in embryonated hens' eggs described below.

Fourth experiment: Because of the possibility that infectious hepatitis virus, like mumps[15, 16] and measles[17, 18] viruses, might lose virulence for humans during prolonged serial egg transfers, a test for the infectivity of an Akiba line started from the serum pool mentioned above was made after only 5 yolk sac passages at ten-day intervals. Each of 6 volunteers was fed a mixture of 6 ml. of yolk sac suspension and 3 ml. of embryo body suspension. All remained well.

Fifth experiment: This experiment was designed to make a more drastic trial of the infectivity of egg passage material. Egg passages were started with serum from Volunteers J. Y. and H. E., as well as from the serum pool from the Philadelphia volunteers. The passage lines and inocula tested are shown in Table I. Seven volunteers fed the multiple egg inocula on Feb. 19, 1953, remained well.

TABLE I. FIFTH EXPERIMENT: INOCULA FED TO SEVEN VOLUNTEERS ON FEB. 19, 1953, FROM EMBRYONATED HENS' EGGS IN WHICH AKIBA INOCULA WERE PASSAGED

INITIAL INOCULUM FOR EGGS		PASSAGES			INOCULUM FED TO VOLUNTEERS	
PATIENTS	SPECIMENS	DATE INITIATED	NO.	ROUTE	TISSUE OR FLUID	DOSE PER MAN (ML.)
Philadelphia volunteers	Pooled serum	11.3.52	5	Yolk sac	Yolk sac (20%)	7
					Embryo (50%)	6
		11.3.52	8	Amniotic	Amniotic fluid	1-1.8
					Embryo (50%)	7
Volunteer J. Y.	Serum	12.12.52	5	Amniotic	Amniotic fluid	0.5
					Embryo (50%)	6
Volunteer H. E.	Serum	12.12.52	5	Amniotic	Amniotic fluid	1.5
					Embryo body	7.0

Sixth experiment: A similar experiment was carried out with the aid of 11 more volunteers who were inoculated on April 24, 1953, with a pool made up of materials harvested after 5 to 7 yolk sac or amniotic sac passages, and on April 27, with twelfth passage material (Table II). As in the preceding trial, egg passage lines for this experiment were originated from the Philadelphia Akiba serum pool and either from fecal or from serum specimens obtained during acute illness from Volunteers J. Y. and H. E., respectively. The latter were promptly frozen on dry ice. Each subject in this experiment was fed a total of approximately 100 ml. of the mixtures of various egg passage materials. All remained well.

Skin Tests With Amniotic Fluid Antigens.—During these studies intradermal tests were done on a total of 23 volunteers with 0.1 ml. doses of various amniotic fluid antigens from JF strain passages, and also with antigens obtained

TABLE II. SIXTH EXPERIMENT: INOCULA FED TO ELEVEN VOLUNTEERS ON APRIL 24 OR APRIL 27, 1953, FROM EMBRYONATED HENS' EGGS IN WHICH AKIBA INOCULA WERE PASSAGED

INITIAL INOCULUM FOR EGGS		PASSAGES			INOCULUM FED TO VOLUNTEERS	
PATIENTS	SPECIMENS	DATE INITIATED	NO.	ROUTE	TISSUE OR FLUID	DOSE PER MAN (ML.)
Philadelphia volunteers	Pooled serum	11. 3.52	5	Yolk sac	Embryo (50%)	4.0-5.5*
		7.21.52	12	Yolk sac	Yolk sac (20%)	20†
					Embryo (50%)	10†
Volunteer J. Y.	Feces	3. 4.53	5	Amniotic	Amniotic fluid	0.3*
					Embryo (50%)	3.5*
		3. 4.53	5	Yolk sac	Yolk sac (20%)	3.0*
					Embryo (50%)	2.0*
	Serum	12.12.52	7	Amniotic	Amniotic fluid	1.5-3.5*
					Embryo (50%)	20*
Volunteer H. E.	Feces	3. 4.53	5	Yolk sac	Yolk sac (20%)	3.5*
					Embryo (50%)	3.0*
	Serum	12.12.52	7	Amniotic	Amniotic fluid	7.0*
		12.12.52	5	Yolk sac	Embryo (50%)	5.0-7.0*

*On April 24.
†On April 27.

through the kindness of Dr. Gertrude Henle. The group included the three men who had developed hepatitis with jaundice during the infectivity titration of the Akiba serum pool.

There was no consistent pattern of positive skin tests. The few that were observed were mostly of the immediate, histamine-like type, and were paralleled by the outcome of simultaneous control injections of fluids from uninoculated eggs.

DISCUSSION

Three mild, atypical illnesses resulted from attempts to induce hepatitis in a total of 49 subjects with inocula derived from 8 infectious hepatitis egg passage lines. They occurred among the 10 men in the first experiment. The incubation periods were disparate; one patient exhibited hepatomegaly and splenomegaly seven days after inoculation and a second developed signs ten days after inoculation. These incubation periods are considerably shorter than the rule in infectious hepatitis. The third incubation period was 30 days. None of the 3 men exhibited multiple abnormalities of liver function tests at a given time. An effort to transmit illness by feeding serum from the ill volunteers to a second set of 10 volunteers was a failure.

The illnesses were milder and less definite than those reported by Drake and associates.[4] Leftwich and co-workers[5] have pointed out that the agents they carried in chick embryo passages were not proved to be IH virus, although their identity with IH virus has not been definitely excluded. They and Henle[1] have commented on factors influencing the interpretation of trials such as the ones reported here, noting that titrations in humans of the infectivity of inocula such as the Akiba serum pool can give discrepant results on different occasions. We succeeded in inducing hepatitis with jaundice in 2 of 4 volunteers fed a 1:100 dilution and in 1 of 5 men fed a 1:1,000 dilution of the pool. Henle and his colleagues,[19] however, were unable to infect volunteers with

aliquots of the same pool; neither a 1:100 dilution (8 men) nor a 1:1,000 dilution (8 men) induced hepatitis. It is suggested[5] that the discrepancy between their results and ours might have been due to antibodies present in the pool. When individual component sera of the same pool were fed consecutively by Leftwich and associates[5] to their subjects, hepatitis resulted. The same was true when a similar inoculation scheme was later followed in other experiments. Leftwich and associates[5] showed that volunteers who developed mild illness after swallowing chick embryo inocula did not resist challenge with the Akiba strain. Tests of the immunizing capacity of IH egg passage lines by challenge with natural virus are severe, however, and the failures are not clear evidence that the egg lines contained no virus. Even when overt disease is induced with virus from human sources, recovered volunteers are not always resistant to challenge.[20, 21]

The results of our trials of the infectivity of the Akiba serum pool, and also the fact that we tested a relatively large number of men, do weigh against the assumption that pre-existing immunity of the volunteers was responsible for the negative outcome of all of our experiments with egg inocula from the second one on. Neither are there obvious technical faults that might explain the results. The amniotic passages were done in much the same way as in the investigations reported by Henle and co-workers,[3] and the yolk sac passages for our later attempts were carried out by the procedures employed in our own encouraging first experiment. Our fifth and sixth experiments, in particular, might have been expected to result in cases of hepatitis. Like the fourth experiment, the inocula given the volunteers included egg lines at relatively low passage levels, but carry-over of viable original inoculum, which might have accounted for the disease reported by Essen and Lembke[6] in volunteers given third or fourth passage allantoic fluid, apparently did not occur in eggs of our fifth or sixth serial transfers. Furthermore, our fifth and sixth experiments were done with egg lines initiated with fecal or serum specimens collected and stored under optimum conditions, and a total of 18 men were heavily inoculated with a variety of materials from a number of different passage lines.

It is impracticable to study infectious hepatitis in the embryonated hen's egg if there is no better way of recognizing infection than to inoculate volunteers. Verification of reports that IH infection of embryonated hens' eggs can be detected by electron microscopy[6, 22] or by effects of the agent on the host[23] would solve the problem, but as yet these studies have not been independently confirmed. The results of skin tests with egg antigens are irregular.[1] The embryonated hen's egg seems of little value for further investigations of infectious hepatitis at present. Until new methods are devised or existing ones improved, studies of the disease by use of this host are likely to remain seriously handicapped.

SUMMARY

The purpose of this study was to evaluate the embryonated hen's egg as a host for the propagation of the agent of infectious hepatitis. Serum hepatitis (hepatitis B) was not studied.

Serum or fecal specimens from naturally or experimentally infected patients with infectious hepatitis were injected into the amniotic or yolk sacs of embryonated hens' eggs and serial transfers were carried out by these routes. Volunteer subjects were fed various egg inocula. In the first experiment attempted, 3 of 10 men who swallowed yolk sac passage inoculum developed mild illnesses after incubation periods of 7, 10, and 30 days, respectively. Their clinical manifestations were not typical of infectious hepatitis but no other cause of illness was identified. Ten men fed pooled serum from the 3 ill volunteers remained well.

In five subsequent experiments carried out with the aid of 39 other volunteers, attempts to confirm this result failed. The inocula included the same egg passage line employed in the first experiment, and 7 others. The infectivity of the serum pool used to initiate some of the egg passages was proved by volunteer inoculation; 1:100 and 1:1,000 dilutions of the pool induced typical hepatitis with jaundice. Fecal and serum specimens collected from 2 of the induced cases provided excellent starting inocula for the rest of the egg passages. There were no obvious technical factors to account for the failure to confirm the outcome of the first experiment.

In these studies the embryonated hen's egg was of little value as a host for investigation of infectious hepatitis. The limitations and deficiencies of this host are probably sufficient to demand development of new techniques of using it or discovery of new approaches to the problem.

The volunteer experiments were carried out at the New York State Vocational Institution, West Coxsackie, New York. They were possible only because of the generous interest and wholehearted support of Donald D. Scarborough, Superintendent; Joseph P. Conboy, then Assistant Superintendent; Dr. Solomon Yarvin, Senior Physician; and their respective staffs. We take pleasure in acknowledging our indebtedness to Edward J. Donovan, formerly Commissioner of the New York State Department of Correction; to William Leonard, Deputy Commissioner, who made many of the necessary arrangements; and to Leroy Weaver, Superintendent of the Elmira Reformatory, and Dr. M. E. Pittman, Senior Physician, and their respective staffs, for their consistent helpfulness.

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STUDIES ON GAMMA GLOBULIN

I. Distribution and Metabolism of Antibodies and Gamma Globulin in Hypogammaglobulinemic Patients

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THE syndrome of hypogammaglobulinemia (or agammaglobulinemia), like other diseases associated with isolated defects of plasma protein synthesis, such as hemophilia, afibrinogenemia, and analbuminemia, offers an opportunity to investigate in man the detailed metabolism of the specific missing plasma protein.

Previous studies in this laboratory on a patient with acquired hypogammaglobulinemia suggested that individual human antibodies are passively degraded at different rates,[1] a phenomenon consistent with recent chemical demonstrations of subtle structural differences between individual species of antibody in electrophoretically and antigenically "homogeneous" preparations of gamma globulin.[2, 3]

This paper presents the results of measurements in 4 hypogammaglobulinemic patients of the passive rates of decline of pooled human gamma globulin and of 7 common antibodies derived from it, together with observations on the distribution of gamma globulin in body fluids.

HYPOGAMMAGLOBULINEMIC PATIENTS

Case 1.—E. W., 13 years of age, Negro, male. Beginning at 18 months of age, upper respiratory infections, pneumonia with empyema and bacteremia, acute otitis media, and bronchitis each developed repeatedly, and 2 episodes of septic arthritis occurred. At the time of study there was evidence of moderate bilateral bronchiectasis, chronic pansinusitis, chronic left otitis media, and hepatosplenomegaly.

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Case 2.—K. M., 36 years of age, white, female. Following a severe pneumococcal lobar pneumonia at age 30, there developed 3 episodes of pneumonia with bacteremia, a severe wound infection, appendicitis with rupture, generalized herpes zoster, exacerbation of a chronic psoriasis-like skin disease present earlier in life, and repeated attacks of sinusitis, bronchitis, pharyngitis, and cutaneous furunculosis. While under study, the patient was found to have hepatosplenomegaly, early bilateral bronchiectasis, chronic frontal and maxillary sinusitis, and, scattered over the neck, arms, trunk, and extremities, numerous painful, scaly skin plaques, with an associated serous exudate.

Case 3.—M. O., 54 years of age, white, female. Following an episode at age 40 of septic arthritis and bacteremia due to *Streptococcus pyogenes*, there developed 11 attacks of pneumonia and bronchopneumonia (at least 5 associated with bacteremia due to *Diplococcus pneumoniae*), staphylococcal septicemia, pneumococcal meningitis and otitis media, generalized herpes zoster, and innumerable episodes of cellulitis, sinusitis, pharyngitis, and bronchitis. Studies disclosed severe bilateral bronchiectasis, pansinusitis, and hepatosplenomegaly.

Case 4.—R. H., 64 years of age, white, female.* Beginning insidiously when the patient was age 55, repeated upper respiratory infections, a mediastinal mass, recurrent bronchitis and "grippe," 12 to 14 pneumonias and bronchopneumonias, and several episodes of pyelonephritis occurred. Studies disclosed a stable mediastinal mass (presumably a hyperplastic thymus), chronic pyelonephritis, mild bronchiectasis, hepatosplenomegaly, a moderate hemolytic anemia, chronic sinusitis, and arteriosclerotic heart disease, with moderate ankle edema despite therapeutic doses of digitoxin.

In none of the 4 patients was there any evidence of cirrhosis, nutritional deficiency, or endocrine or neoplastic disease, and none exhibited proteinuria, pleural effusion, or ascites.

MATERIALS AND METHODS

Each patient was given parenterally 250 to 300 ml. of pooled human gamma globulin† over a 1- to 12-day period (Table I), and serum levels of gamma globulin and antibodies were followed for 75 to 80 days. The blood sample representing the peak serum level of passively acquired gamma globulin was drawn 4 to 9 days after the last of the series of gamma globulin doses. Although daily serum gamma globulin measurements after the last dose suggested virtually complete absorption and equilibration in 2 days of a 50 ml. dose of gamma globulin given intramuscularly in 4 sites, the additional time was allowed in order to minimize any effect of incomplete equilibration.

Clinical Conditions.—Seven to fifty-six days prior to gamma globulin administration, each patient was given a priming dose of sulfadiazine, and was thereafter maintained, over the 90- to 125-day period of study, at a constant serum sulfonamide level of 5 to 9 mg. per 100 ml. The course of chemotherapy effectively eliminated, prior to gamma globulin administration, the major sources of nonspecific protein loss in each patient—pneumonitis (M. O., R. H.), acute sinusitis (M. O., E. W.), draining chronic otitis media (E. W.), and skin eruption (K. M.)—and reduced to a minimum sputum production in the patients with the more severe bronchiectasis (M. O., E. W.).

To rule out the possibility of active infection with any of the bacteria or viruses the antibodies against which were being measured, weekly or biweekly sputum and stool specimens and nose and throat swabs were cultured for bacteria and most specimens inoculated into suckling mice and HeLa cell and monkey-kidney cell roller-tube cultures. At no time were *Streptococcus pyogenes, Salmonella typhi, Clostridium tetani, Corynebacterium diphtheriae* or Coxsackie, adenoidal-pharyngeal-conjunctival, or poliomyelitis viruses isolated.

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*Case history previously reported in detail.[1]

†Poliomyelitis Immune Globulin, Squibb and Sons, kindly supplied by Dr. J. N. Ashworth of the American National Red Cross. R. H. received Lot #236; K. M., #204; and E. W. and M. O., #252. All 3 lots were virtually identical in protein and antibody content and in number, age, sex, time of bleeding, and geographic source of origin of the donors contributing to the original plasma pools.[4]

The patients' weights remained essentially constant throughout the study.

Laboratory Tests.—Serum gamma globulin was measured in an Aminco Model B Tiselius electrophoresis apparatus equipped with a Rayleigh interferometric optical system,[5] the sensitivity of which is approximately that of the equine antiserum immunochemical technique of Gitlin.[6] Sera were dialyzed against a barbital buffer of pH 8.6 and ionic strength 0.1.

Antistreptolysin O antibody was expressed as the mean of 2 measurements by the method of Rantz and Randall,[10] using twofold serum dilutions and choosing 50 per cent hemolysis as the end point. Typhoid H agglutinins were expressed as the mean of 2 measurements by the slide agglutination method of Welsh and Stuart,[11] employing the modifications described in a previous study.[12] Diphtheria antitoxin was measured by the rabbit-skin titration technique,[13] and tetanus antitoxin by the mouse intravenous protection test,[14] employing National Institutes of Health reference antitoxins as the controls.

Tests for viral neutralizing antibodies were performed as follows: against Coxsackie A_{10} (1816, Alaska 5) and A_9 (5955, Boston)—a modification of the suckling-mouse protection test of Beeman and associates,[7] employing a 1,000 ID_{50} challenge dose; against poliomyelitis, Type I (Mahoney) and Coxsackie A_9—the monkey-kidney roller-tube culture protection test, by the method of Shelokov and Habel,[8] using a 50 ID_{50} challenge dose; and against adenoidal-pharyngeal-conjunctival virus, Type 2—the HeLa cell roller-tube culture protection test of Rowe and co-workers.[9] Because of evidence (described below) that tissue culture titration test end points were grossly inaccurate when based on readings at 72 hours after inoculation, end points for the viral neutralizing antibodies were determined from fifth- or sixth-day readings. The 2 or 4 tubes at each twofold dilution were then read as "infected" or "not infected," without regard to degree of cytopathogenesis, and the neutralization titer was expressed as the serum dilution* affording complete protection to 50 per cent of the cultures, as calculated by the method of Reed and Muench.[15]

All antibody tests were performed with both negative and positive controls; in the few instances in which normal rabbit serum possessed slight nonspecific inhibitory activity, serum from patient M. O., drawn prior to gamma globulin therapy, served as a satisfactory negative serum both in 1:1 and in higher dilutions. All specimens from a given patient for one test were run at one time. Sera were kept frozen in the interval between collections and testing.

Statistical Methods.—The exponential curves of decline of gamma globulin and antibodies in each patient were expressed as the slopes of the straight regression lines calculated by the method of least squares. The arithmetic mean half-life, standard deviation, and standard error for each antibody were calculated from the half-life values in each patient, and the mean half-life of each antibody was compared with that of every other antibody by the "t-test" of Fisher.[16]

Compartment Size.—The apparent volume of distribution of gamma globulin in each patient was determined as follows: the straight regression line of passive exogenous gamma globulin decline was extrapolated to Day O, the exogenous serum gamma globulin level on Day O subtracted from this figure, and the total dose divided by the difference (Table I).

RESULTS

Studies of compartment size are summarized in Table I. Excluding patient R. H., whose excessively large compartment size presumably reflects her expanded plasma and interstitial fluid volumes due to her moderate anemia and congestive heart failure, the mean compartment size in the group was 11.8 ± 1.5 per cent of body weight. Compartment size in these 3 patients also bore approximately the same relationship to surface area—3.7 ± 0.5 liters per square meter.

*Before the addition of virus suspension and tissue culture medium.

TABLE I. DISTRIBUTION OF POOLED HUMAN GAMMA GLOBULIN IN 4 HYPOGAMMAGLOBULINEMIC PATIENTS

	CONGENITAL		ACQUIRED	
Patient	E. W.	K. M.	M. O.	R. H.
Age	13	36	54	64
Weight (Kg.)	40.8	49.5	42.4	45.0
Surface Area (meter²)	1.33	1.56	1.42	1.43
Endogenous Serum γ-globulin*	.030	.030	.027	.110
Half-life, Exogenous γ-globulin (days)	32.1	30.8	31.6	36.0
Total γ-globulin dose (Gm.)	46.8	39.25	46.8	48.6
Method of Administration	7.8 Gm. i.m., 4 sites, Days 0, 2, 6, 8, 10, 12.	7.85 Gm. i.m., 4 sites, Days 0, 2, 4, 6, 8.	7.8 Gm. i.m., 4 sites, Days 0, 2, 4, 6, 8, 10.	24.3 Gm. s.c., each thigh, Day 0.
Exogenous Serum γ-globulin,* Day 0	.050	.170	.021	.040
Peak Exogenous Serum γ-globulin* after equilibration, and Day of Peak	.827 (Day 16)	.575 (Day 13)	.682 (Day 15)	.440 (Day 9)
Exogenous Serum γ-globulin,* Extrapolated to Day 0	1.168	.770	.948	.491
Compartment Size (liters)	4.19	6.54	5.05	10.8
Compartment Size (per cent of Body Weight)	10.3	13.2	11.9	24.0
Compartment Size per Surface Area (liters per meter²)	3.2	4.2	3.6	7.6

*Gram per 100 ml.

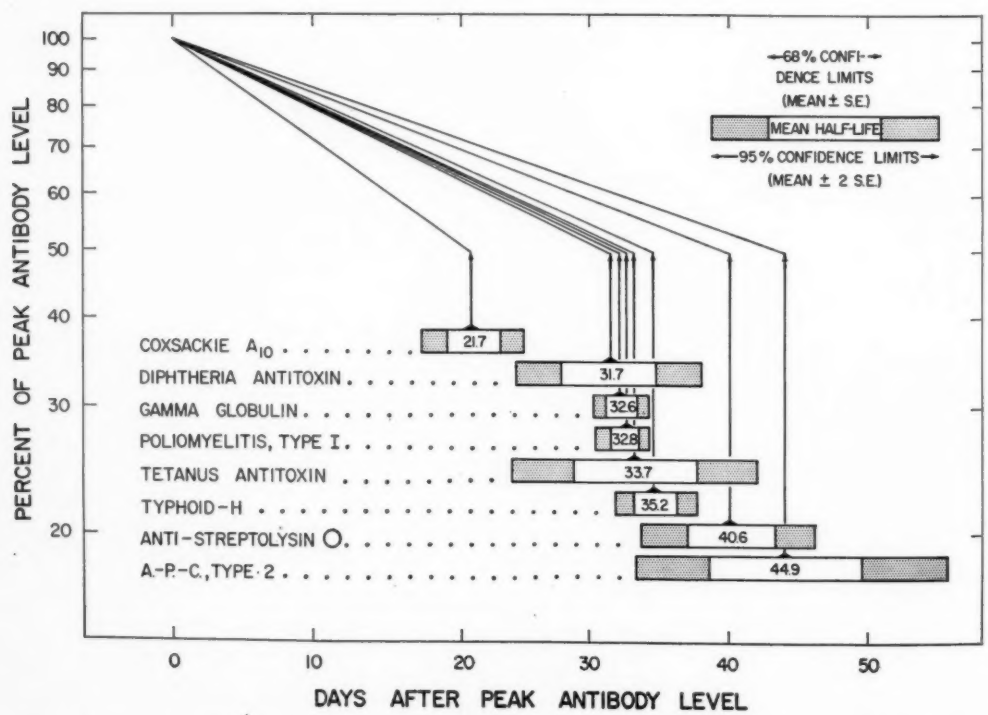

Fig. 1.—Mean rates of decline of passively acquired antibodies in 4 hypogammaglobulinemic patients.

The results of studies of the passive decline of antibodies and of gamma globulin are summarized in Table II and illustrated in Fig. 1. Mean antibody half-lives ranged from 21.7 ± 3.7 days (Coxsackie A_{10} neutralizing antibody) to 44.9 ± 11.7 days (APC Type 2 neutralizing antibody).

The portrayal in Fig. 1 of both the 68 per cent and the 95 per cent confidence limits of the mean half-lives of gamma globulin and of individual antibodies illustrates graphically the results of the "t-tests": the mean half-life of Coxsackie A_{10} neutralizing antibody was significantly different ($P < .01$) from the half-lives of all the others except those of diphtheria and tetanus antitoxin, from which it was probably significantly different ($.01 < P < .05$).* None of the remaining mean half-lives was significantly different from any other. In individual patients, however, multiple differences were demonstrable at both the $P < .01$ and the $P < .05$ levels of significance between the half-lives of several antibodies. The half-life of patient K. M.'s exogenous gamma globulin, for example, was significantly different ($P < .01$) from that of both her APC and her antistreptolysin O antibodies.

Because of the seemingly anomalous behavior of Coxsackie A_{10} antibody, experiments were performed to preclude the possibility of any fundamental inaccuracy in the method of testing which would yield an erroneously short half-life.

Six sera of known relative antibody content (mixtures in varying proportion of antibody-containing serum of patient E. W., drawn at the peak of the gamma globulin curve, and known negative, pre-gamma globulin serum of patient M. O.) were made up and assigned time values so as to represent points on a hypothetical straight regression line with a half-life of 30 days. Upon titration of the sera in suckling mice, the observed values yielded a regression line with a half-life of 32.2 days.

In addition, the passive half-life in one patient of neutralizing antibody against Coxsackie A_9, the only A-strain which produces a cytopathogenic effect upon monkey-kidney cells,[17] was measured both in suckling mice and in monkey-kidney roller-tube cultures (Table II). The observed half-lives were 24.0 days in tissue culture and 20.5 days in mice.

From these data it was concluded that measurements of Coxsackie A_{10} antibody half-life in suckling mice reflected no unduly large consistent errors.

Because measurements of the half-life of neutralizing antibodies in roller-tube tissue cultures were found to vary strikingly according to the day of reading of the cultures and the method of choosing an end point in a titration, the 6 sera of known relative antibody content described above were titrated in monkey-kidney cultures for Coxsackie A_9 and Poliomyelitis Type I

*As a cross check on the statistical methods employed, a short variance analysis of the data in Table II was performed with the advice and assistance of Dr. Hugo Muench, Professor of Biostatistics, Harvard University School of Public Health, who kindly reviewed the manuscript. For 3 patients and 7 antibodies, the variance (Σ squares) was distributed as follows: Total: 1,480.66; Patients: 132.49; Antibody mean half-lives: 1,250.09; Residual (experimental error, patient-antibody interaction): 98.08. With 20, 2, 6, and 12 degrees of freedom, respectively, it is concluded that there is a significant difference in the mean half-lives of the various antibodies; that there is a significant difference between the patients; and that, as evidenced by the small residual variance, the patients' over-all handling of different antibodies appears to be homogeneous.

TABLE II. LEVELS AND HALF-LIVES OF PASSIVELY ACQUIRED POOLED HUMAN GAMM

ANTIBODY		DAYS AFTER PEAK 0	7	14	15	21	27	28	29	35	42	43	SERUN 49
A.P.C., Type 2 Neutralizing Antibody*	E. W.	22.4	—	—	—	11.2	—	—	—	6.4	—	—	—
	K. M.	11.2	—	—	10.6	—	—	—	—	—	—	5.3	—
	M. O.	11.2	—	—	—	9.3	—	—	—	—	6.9	—	—
	R. H.	12.0	—	—	—	—	—	—	—	—	5.0	—	—
Antistreptolysin O Antibody*	E. W.	64	48	—	—	48	—	—	—	40	—	—	24
	K. M.	80	—	—	64	—	—	—	48	—	—	40	—
	M. O.	80	—	64	—	—	—	40	—	—	32	—	—
	R. H.	—	—	—	—	—	64	—	—	—	40	—	40
Typhoid H Agglutinin*	E. W.	50	40	—	—	30	—	—	—	20	—	—	20
	K. M.	17.5	—	—	12.5	—	—	—	10	—	—	7.5	—
	M. O.	35	—	30	—	—	—	17.5	—	—	15	—	—
	R. H.	10	—	—	—	—	<10	—	—	—	<10	—	—
Tetanus Antitoxin, Units Per Milliliter	E. W.	.75	—	—	—	.25	—	—	—	—	—	—	.125
	K. M.	.016	—	—	.016	—	—	—	—	—	—	.008	—
	M. O.	.25	—	—	—	—	—	—	—	—	.125	—	—
	R. H.	.063	—	—	—	—	.031	—	—	—	.031	—	—
Poliomyelitis, Type I Neutralizing Antibody*	E. W.	15.8	—	—	—	12.2	—	—	—	13.0	—	—	—
	K. M.	6.6	—	—	—	—	—	—	5.0	—	—	3.3	—
	M. O.	12.6	—	—	—	—	—	5.3	—	—	—	—	5.6
Exogenous Gamma Globulin, Grams Per 100 Milliliter	E. W.	.90	.68	—	—	.505	—	—	—	.37	—	—	.29
	K. M.	.54	—	—	.445	—	—	—	.30	—	—	.22	—
	M. O.	.55	—	.46	—	—	—	.325	—	—	.24	—	—
	R. H.†	.44	.38	—	—	.23	—	.24	—	.21	.20	—	—
Diphtheria Antitoxin, Units Per Milliliter	E. W.	.32	—	—	—	.08	—	—	—	—	—	—	.08
	K. M.	.08	—	—	.06	—	—	—	—	—	—	.04	—
	R. H.	.15	—	—	—	—	—	—	—	—	.04	—	—
Coxsackie A_{10} Neutralizing Antibody*	E. W.	31.6	31.6	—	—	3.2	—	—	—	10	—	—	5.6
	K. M.	12.6	—	—	20.6	—	—	—	3.2	—	—	2.1	—
	M. O.	17.8	—	10	—	—	—	7.1	—	—	3.2	—	—
	R. H.	31.6	—	—	—	—	5.6	—	—	—	3.2	—	—
Coxsackie A_9 Neutralizing Antibody*													
Tissue Cult.:	E. W.	50.8	—	—	—	22.4	—	—	—	15.8	—	—	—
Mice:	E. W.	31.6	10	—	—	3.2	—	—	—	3.2	—	—	3.2

*Reciprocal of titer.

†Exogenous serum gamma globulin .38, .37, .35, and .18 on Days 3, 10, 13, and 48 after the peak.

‡Calculated with the correction for small sample size.

neutralizing antibodies. Readings of the cultures at 72 and at 96 hours after inoculation consistently yielded values which maximized nonspecific neutralizing or infection-delaying effects, resulting in regression lines with half-lives of 45 to 75 days. Half-lives of 26 to 33 days, the closest to the hypothetical 30-day half-life, were obtained only by permitting the test to run its full 5- or 6-day course[18, 19] and expressing the titer of each specimen as the dilution affording complete protection to 50 per cent of the cultures.

DISCUSSION

The finding that pooled human gamma globulin is distributed in hypogammaglobulinemic patients in an apparent fluid volume of approximately 12

GAMMA GLOBULIN AND ANTIBODIES IN 4 HYPOGAMMAGLOBULINEMIC PATIENTS

SERUM LEVEL								HALF-LIFE (DAYS)	MEAN HALF-LIFE (DAYS)	STANDARD DEVIATION‡ (DAYS)	STANDARD ERROR OF MEAN (DAYS)
49	55	56	57	63	66	69	70				
—	—	—	—	5.6	—	—	—	31.3			
—	—	—	—	—	6.0	—	—	59.6	44.9	11.7	5.842
—	—	—	—	—	—	—	4.0	46.4			
—	—	—	—	—	—	4.0	—	42.4			
—	24	—	—	—	20	—	—	—	39.1		
40	—	—	—	40	—	24	—	—	44.5	40.6	6.6	3.3115
—	—	—	20	—	—	—	20	31.9			
—	40	—	—	40	—	—	32	—	46.8		
—	20	—	—	—	15	—	—	—	37.5		
7.5	—	—	—	<7.5	—	<7.5	—	—	35.8	35.2	2.6	1.4994
—	—	—	12.5	—	—	—	—	7.5	32.4			
—	—	<10	—	—	—	—	<10	—	—			
—	.125	—	—	—	.125	—	—	—	24.3			
.008	—	—	—	—	—	.004	—	—	31.5	33.7	8.9	4.4700
—	—	—	—	—	—	—	—	.063	33.3			
—	—	—	—	—	.031	—	.016	—	45.8			
—	—	—	—	—	3.7	—	—	—	30.9			
3.3	—	—	—	—	—	1.7	—	—	34.3	32.8	1.7	1.0087
—	5.6	—	—	—	—	—	—	2.5	33.3			
—	.29	—	—	—	.22	—	—	—	32.1			
.22	—	—	—	.156	—	.130	—	—	30.8	32.6	2.1	1.0600
—	—	—	.156	—	—	—	—	.13	31.6			
—	—	.14	—	—	.15	—	.12	—	36.0			
—	.08	—	—	—	.08	—	—	—	35.9			
.04	—	—	—	—	—	.02	—	—	34.3	31.7	5.9	3.3984
—	—	—	.04	—	—	—	.02	—	25.0			
—	5.6	—	—	—	3.2	—	—	—	20.6			
2.1	—	—	—	2.1	—	3.2	—	—	22.3	21.7	3.7	1.8530
—	—	—	3.2	—	—	—	—	3.2	26.3			
—	—	3.2	—	—	—	—	1.8	—	17.4			
—	—	—	—	—	8.0	—	—	—	24.0	22.3	2.5	1.7503
—	3.2	—	—	—	2.5	—	—	—	20.5			

per cent of body weight accords with demonstrations by Gitlin and co-workers of a gamma globulin compartment of similar size in animals.[20, 21] Assuming that the plasma volume is close to 5 per cent of body weight, then, as in the case of albumin[22, 23] and fibrinogen,[24] roughly 40 to 45 per cent of the gamma globulin pool is intravascular, and the remainder is widely distributed in cells and interstitial fluids.[25]

The 32.6-day mean half-life of pooled human gamma globulin (31.5 days, if patient R. H. is excluded) is in substantial agreement with recent half-life measurements in normal subjects, with the use of autogenously-biosynthesized radioactive S^{35}-labeled gamma globulin.[26, 27] The shorter (9- to 20-day) half-life values obtained in normal subjects with radioactive I^{131}-labeled gamma globulin[28-30] are difficult to compare in view of several recent technical objections to the I^{131} method.[22, 26, 31, 32]

Although the number of subjects was small and the patients possibly unrepresentative, the roughly inverse relationship described by others between age of the subject and gamma globulin half-life[28] was not verified. In this regard, too, the 31.7-day mean half-life of diphtheria antitoxin in the group agrees closely with Wiener's estimate of 30 days as the half-life of placentally transmitted diphtheria antitoxin in newborns.[33]

The demonstration of different rates of antibody decline in individual patients (Table II) confirms earlier findings in this laboratory.[1] The relatively large pooled standard deviation of both the in vivo half-life determinations (17.4 per cent) and the in vitro determinations (10.0 per cent), however, emphasizes the problem of demonstrating statistically significant differences among closely grouped mean half-lives and suggests that 4 to 16 times as many half-life measurements would be necessary to demonstrate further differences.

That differences are demonstrable at all, however, with gamma globulin containing specific antibody with the distribution of properties to be expected in a pool derived from upwards of 25,000 donors, strongly suggests not only that individual species of antibody are degraded at different rates in the same individual but that the same species of pooled antibody is degraded at approximately the same rate in different individuals.

These findings therefore imply that at least a part of the variation in gamma globulin half-life noted in studies with S^{35}-labeled gamma globulin[26, 27] may be attributed to a skewing effect in favor of the specific half-life of the preponderant antibody being synthesized at the time of isotope incorporation.

Studies of the mechanism of the phenomenon of differing antibody half-lives are in progress in this laboratory. Preliminary experiments with different systems suggest that pooled human gamma globulin can be fractionated in vitro and its component antibodies segregated in the order of their electrophoretic mobilities, an order which tends to correlate with the mean half-lives of the antibodies in vivo.[34]

SUMMARY

Studies in 4 hypogammaglobulinemic patients indicate that injected pooled human gamma globulin is distributed in an apparent fluid volume of approximately 12 per cent of body weight and that in each patient individual antibodies derived from the pool are degraded at different rates.

All 4 patients degraded each antibody at approximately the same rate. The mean half-lives of individual antibodies in the group varied from 21.7 to 44.9 days, while that of pooled gamma globulin was 32.6 days. The implications of these findings are discussed.

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LABORATORY METHODS

A PHYSIOLOGICALLY ORIENTED ARTIFICIAL RESPIRATOR WHICH PRODUCES N_2O–O_2 ANESTHESIA IN MAN

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THIS automatic device produces anesthesia and artificial respiration for surgical patients immobilized by the skeletal muscle relaxant, succinylcholine. Pulmonary ventilation is carried out with an N_2O–O_2 mixture of fixed composition which produces both anesthesia and oxygenation. The artificial respiration is carried out on a principle closely simulating the ordinary control of breathing. Normally, the respiratory centers of the brain stem maintain the arterial CO_2 tension at a fixed level by regulating the rate and depth of breathing. This artificial respirator monitors the end-tidal (and hence usually also the arterial) CO_2 tension and automatically varies the pulmonary inflating pressure accordingly to maintain the CO_2 tension at the desired level.

METHODS

Preoperative medication usually consisted of only scopolamine. Anesthesia is induced with an intravenous injection of approximately 0.2 Gm. of thiopental. Immobilization and apnea are produced then and maintained for the entire surgical operation by a continuous intravenous infusion of 0.5 per cent succinylcholine chloride in a 0.9 per cent NaCl solution. A tracheal catheter is inserted and an airtight seal obtained with an inflatable cuff. The lungs are then intermittently inflated automatically through this catheter with the N_2O–O_2 mixture (Fig. 1).

Mixer (Fig. 2).—The N_2O and O_2 are delivered at approximately 50 p.s.i. from large capacity reservoirs to a specially designed mixer which blends the gases, fixing the N_2O/O_2 ratio at any desired value. Both N_2O and O_2 enter regulators for delivery at approximately 37 p.s.i. to restrictions leading into the common mixing chamber. The constancy of the composition of the mixture is determined by the stability of the input pressures and the fixed size of the restrictions. However, the N_2O/O_2 ratio can be set quite easily at any desired value by merely turning the knob controlling the size of the restriction for each gas. Usually a 3:1 mixture is employed. A Model D Pauling-Beckman oxygen analyzer was modified for continuous monitoring of this ratio.

The output of this mixing chamber then is delivered in a relatively reproducible and steady fashion into a 20 liter storage tank through a solenoid valve actuated by a pressure-sensitive control on the tank. This solenoid valve opens when the tank pressure falls to

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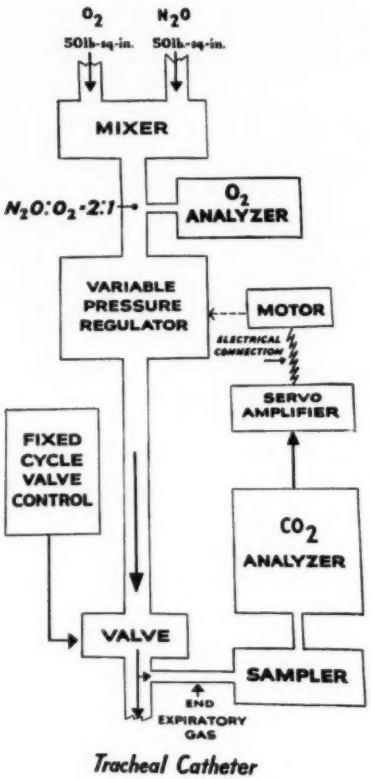

Fig. 1.—Schema of automatic respirator servo controlled according to CO_2 concentrations of the gas at the end of expiration.

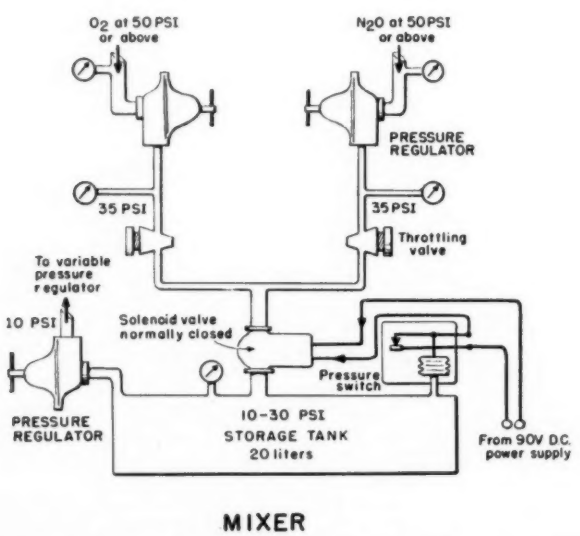

Fig. 2.—Regulator No. 8951, Air Reduction Co., Jersey City, N. J.; Pressure Switch No. L404A2 Pressuretrol, Minneapolis Honeywell Co., Minneapolis, Minn.; Solenoid Valve No. 82621, Automatic Switch Co., East Orange, N. J.

12 p.s.i. and closes when the tank pressure rises to 28 p.s.i. The final or variable pressure regulator operates best when its input pressure is about 10 p.s.i. Therefore, the tank pressure which varies between 12 and 28 p.s.i. is further reduced to this optimal 10 p.s.i. by an intermediary pressure regulator for delivery to the variable low pressure regulator.

Delivery of the Gas Mixture to the Patient (Figs. 3 and 4).—The final stage of pressure control is the variable low pressure regulator which reduces the pressure of the gas mixture from 10 p.s.i. to that used for inflating the patient's lungs. The gas mixture at this low pressure is delivered to the tracheal catheter through a specially designed low resistance 3-way nonrebreathing valve. This valve is pneumatically actuated through a remote-control system which opens and closes it on a fixed cycle determined by a cam rotated by a 15 r.p.m. motor. Inspiration occurs for one-third of the cycle, when this valve opens to permit the gas mixture to enter the tracheal catheter. Exhalation is a passive act occupying the balance of the cycle when the catheter is connected to atmosphere.

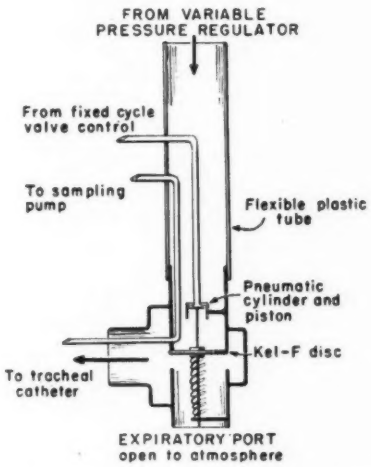

Fig. 3.—Breathing valve in the expiratory position.

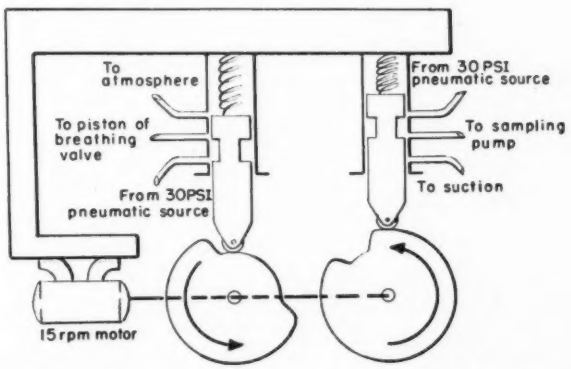

Fig. 4.—Fixed cycle control for breathing valve and sampling pump; K Motor, Bodine Motor Co., Chicago, Ill.

Control of Inflating Pressure (Figs. 5, 6, and 7).—The inflating pressure is the only controlled variable of this respirator. It is automatically adjusted to maintain the end-tidal CO_2 concentration at a predetermined level. The end-tidal sample is obtained by way of a small caliber sampling tube in the lumen of the tracheal catheter which leads to

a 10 ml. single stroke pump. A small filter is interposed in this tube to remove any particulate matter which might otherwise enter the pump. This pump is pneumatically actuated in a manner similar to the 3-way valve and is operated synchronously with the

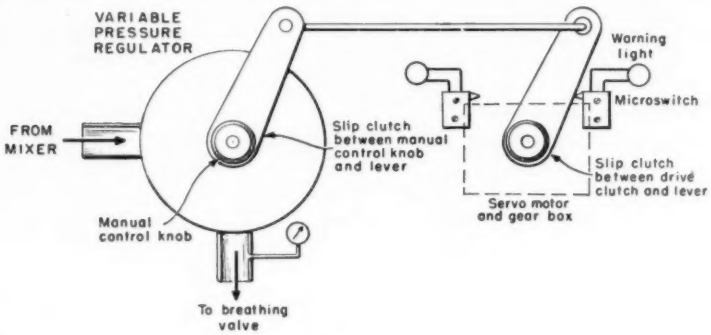

Fig. 5.—Variable pressure regulator and servo motor; Automat Regulator, Ohio Chemical Co., Madison, Wis.

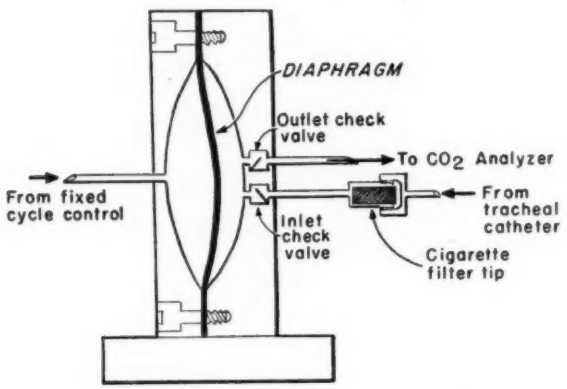

Fig. 6.—Sampling pump in movement.

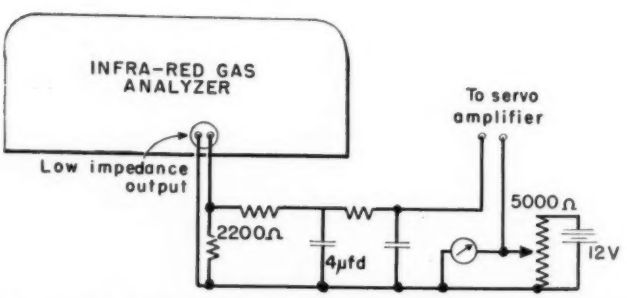

Fig. 7.—Infrared Gas Analyzer No. 16, Beckman Instruments Co., Liston-Becker Division, Springdale, Conn.

valve by means of a second cam on the axle of the 15 r.p.m. motor. This pump samples gas during the last 0.2 second of the expiratory phase of the cycle. If the patient is apneic and does not breathe spontaneously, a valid end-tidal sample is obtained. However, under certain circumstances such as emphysema, thoracotomy, etc., the tensions of CO_2 in the arterial blood and this end-tidal sample will not be identical. This sample is delivered

from the pump to an infrared gas analyzer which carries out the CO_2 analyses. The output of this CO_2 analyzer is then utilized in a servo system which adjusts the setting of the variable pressure regulator controlling the depth of respiration and hence the amount of CO_2 exhaled. For example, if the end-tidal CO_2 concentration exceeds the value which the system is set to maintain, then the inflating pressure is automatically increased, deeper respirations ensue, and more CO_2 is exhaled. These adjustments of approximately 0.1 mm. Hg pressure change per breath continue until the patient's CO_2 concentration matches the setting of the servo.

Numerous safety features have been incorporated into this apparatus and constitute an integral part of its functioning. The drive between the servo motor and the low pressure variable regulator is protected by sets of slip clutches and mechanical stops. The maximum variation which the servo can impose on the variable pressure regulator without manual reset is about 4 mm. Hg. Should any part of this sampling, analysis or readjustment system fail to function correctly, the machine cannot change the inflating pressure by more than this amount from a setting previously adjudged to be correct by the anesthesiologist.

RESULTS

The anesthesia for 171 surgical procedures was carried out entirely with this apparatus.[1] The regulation of the inflating pressure was accomplished through the CO_2 controlled servo system in 64 cases. Initial experimental mixer systems provided oxygen concentrations which varied as much as 10 per cent or more. With the present mixer system, however, the variation in O_2 concentration from the desired value rarely exceeds 1 per cent, the reading error of the analyzer, over many hours of operation. The three-way valve has operated over 300 hours without mechanical failure.

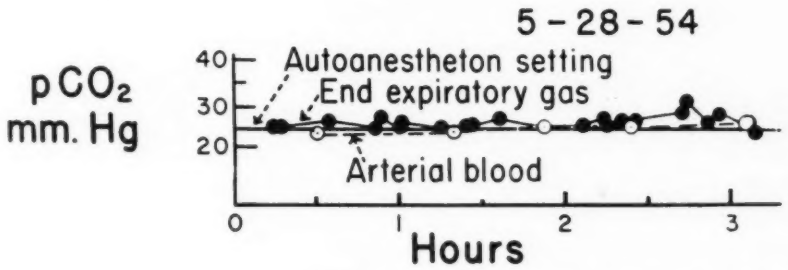

Fig. 8.—Correlation between setting of the automatic respirator, end expiratory CO_2 tension, and arterial CO_2 during a three-hour operation.

The influence of this respirator upon the arterial blood gas concentrations and upon the circulation together with the methods of analyzing will be reported in detail in the future.* Fig. 8 demonstrates the close correlation between the arterial pCO_2 and the CO_2 tension at which this apparatus was set to operate in one patient during three hours of operation. The arterial O_2 saturation in this patient varied between 90 and 95 per cent. The inspiratory oxygen concentrations which were produced in this instance by an early

*We are indebted to Dr. Duncan A. Holaday for collaboration in carrying out arterial pH and gas analyses. Misses M. Verosky and I. Weiss, and Mr. W. Luke provided valuable technical assistance.

experimental mixer system varied between 25 and 30 per cent. The alveolar-arterial O_2 gradient rarely exceeded 30 mm. Hg in 13 subjects studied with the present model of the apparatus.

DISCUSSION

The features of reliability and reproducibility of this artificial respirator and anesthetic gas mixer provide a decided advantage over manual methods of artificial respiration and those systems in which dependence is placed upon the reliability of operation of the conventional anesthesia machine. The assessment and immediate correction of errors in CO_2 exchange are features which duplicate normal breathing and are not used in other existing anesthesia machines. This is particularly important in view of the numerous reports regarding respiratory acidosis during anesthesia first reported by Crafoord.[2]

This method artificial respiration may be useful in other situations in which patients are unable to breathe spontaneously. Saxton and Myers[3] devised an analogous system for ventilating poliomyelitic patients and have aptly described it as an "electromechanical substitute for the human respiratory center." It also may be used experimentally to maintain subjects in predetermined fixed states of hypercapnea or hypocapnea.

SUMMARY

A device is described for providing artificial respiration in man with a fixed mixture of N_2O and O_2. The inflating pressures for the artificial respiration are servo controlled according to the end-tidal CO_2 concentrations, so as to maintain the arterial pCO_2 at a desired level.

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THE ROLE OF THE NITRITE CONCENTRATION IN THE ESTIMATION OF ANTIPYRINE

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ANTIPYRINE is used widely in the measurement of total body water. In the original antipyrine method of Brodie,[1] the spectrophotometric reading of the deproteinized plasma was said to remain constant for a period of 15 minutes beginning 20 minutes after the addition of nitrite. In our hands, however, such constancy was the exception rather than the rule. In fact, in the majority of samples optical density decreased rather rapidly instead of maintaining a plateau. A number of investigators have experienced inconsistencies with the method[2] and there is at least one published account[3] of such difficulties.

EXPERIMENTAL

An attempt, therefore, was made to stabilize the readings by varying the concentration of H_2SO_4 and of the $NaNO_2$ solution, respectively. Changes in acid concentration were not effective in altering the density-time graph but varying the nitrite concentration did so consistently. The higher the nitrite concentration, the sooner the maximum density was attained and the more rapid was the subsequent decrease in density.

The graphs reproduced in Fig. 1 were obtained with antipyrine solutions whose concentrations were equivalent to plasma filtrate concentrations of 2 γ/ml., 5 γ/ml., and 10 γ/ml. Equal volumes of nitrite solution were added, resulting in final concentrations per milliliter of 23 γ, 45 γ, 68 γ, 90 γ, and 136 γ of $NaNO_2$. The readings were taken at 30° C., but comparable curves were obtained at 20° to 22° C. It is apparent that the per cent decrease of optical density as a function of time is essentially the same for any specific concentration of $NaNO_2$ for the three antipyrine concentrations.

The original antipyrine method requires the addition of "2 drops" of a 0.2 per cent $NaNO_2$ solution to 3 ml. of filtrate after the addition of one drop of H_2SO_4. Depending upon the volume of the drop (.05 to .06 ml.) the final concentration of nitrite may vary by 12 γ per milliliter or more (probable range in final total concentration is 63 to 75 γ per milliliter). In view of the

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marked differences in the optical properties with rather small differences in the volume of the added 0.2 per cent nitrite solution, accurately pipetted amounts are essential.

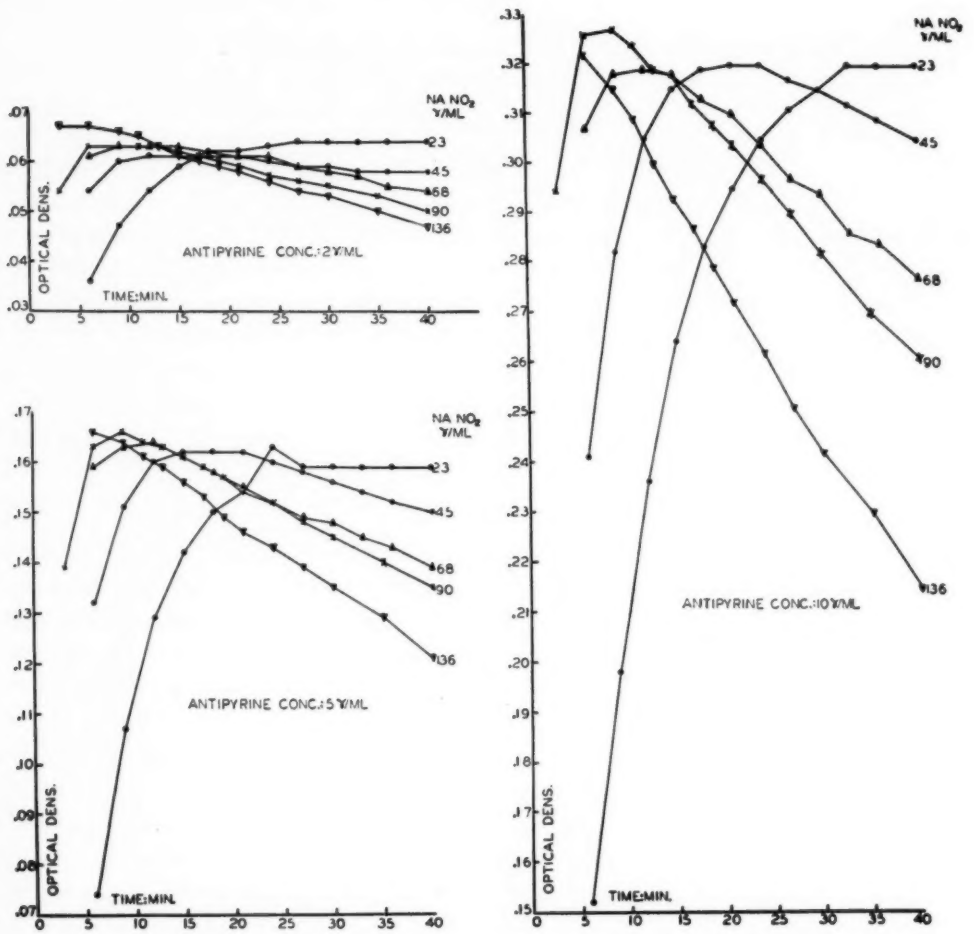

Fig. 1.—Changes of optical density with time following the addition of varying amounts of $NaNO_2$ to acidified antipyrine solutions (2 γ/ml., 5 γ/ml., 10 γ/ml.). Read at 350 mμ and 30° C.

Under the given conditions, a final concentration of 45 γ per milliliter $NaNO_2$ produced a plateau within a reasonable time and exhibited a subsequent slow decrease in optical density. This concentration was chosen therefore as the final concentration of nitrite and consequently the procedure was altered as follows. Fifty λ each of 1.3 N H_2SO_4 and 0.1 per cent $NaNO_2$ solution were used for 1.0 ml. of plasma filtrate. Twenty-three to 28 minutes later readings were taken in a Beckman spectrophotometer at a wavelength of 350 mμ, using a 1.2 ml. capacity silica cell, with a 10 mm. light path. Except for these changes, the Brodie method was adhered to.

SUMMARY

A simple modification of the Brodie method of analysis of antipyrine in plasma is described which improves the method's precision.

ADDENDUM

In a recent article, Davidsson and MacIntyre[4] consider the temperature at which the reaction takes place an essential factor. By using a waterbath at 22° C. for 25 minutes after the addition of nitrite to the plasma filtrate they obtained readings which remained stable for 20 minutes.

It is this author's opinion that the temperature at which the reaction is carried out may well affect the time necessary to attain a plateau, the length of the plateau and the rate of the ensuing fall in optical density. However, within the limits of ordinary room temperature (20° to 30° C.) and under the given conditions, temperature as such or constancy of temperature is unlikely to change materially the basic relationship (Fig. 1) between the different "nitrite concentrations" for a definite antipyrine solution. In our hands, the effects of nitrite concentration outweighed those of temperature, as qualitatively similar results were obtained at room temperatures of 22° C. and 30° C.

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A BIO-ASSAY TECHNIQUE FOR PLASMA ANTIDIURETIC ACTIVITY

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THE uncertainty of results in determinations of the concentrations of the antidiuretic hormone (ADH) reported by several investigators exemplifies the fact that no dependable method is yet available. Problems in the extraction and concentration of the hormone were recognized by Melville.[1] Levitt[2] and Theobold,[3] using ultrafiltrates of plasma, concluded that posterior pituitary hormone previously added to plasma could not be extracted quantitatively. More recently, Teel and Reid[4] reported a bio-assay technique in which the urine of the patient was injected subcutaneously into a hydrated albino rat and the rate of water excretion of the animal measured. By this procedure they demonstrated the presence of antidiuretic activity in the urine of the patients tested. It is possible that in the process of renal excretion, ADH is altered. A change in renal threshold may occur or the associated vasopressin may cause a decrease in urinary excretion so that antidiuretic activity in the urine may not reflect the actual plasma levels of ADH. Thus, the use of unaltered plasma, theoretically, should be a better method than the use of urine or ultrafiltrates of plasma. With these thoughts in mind, and using a modification of the principles of Teel and Reid, we devised a practical bio-assay technique for the determination of antidiuretic activity in plasma.

MATERIAL AND METHOD

Unlike Burns,[5] we found the white female rat an excellent test animal for these determinations. These animals were housed in a controlled temperature laboratory and given free access to food and drinking water. Metabolic cages suitable to our needs were not available; therefore, collection cages were made to suit the requirements of this technique. The bottom was removed from a gallon glass jug. The jug was then inverted and supported on a ring stand. A large mesh wire screen was placed four inches from the mouth of the jug to support the animal, and a fine mesh screen, funnel shaped, was placed at the neck of the jug to collect the feces. The urine flowed down the sides of the jug through a glass funnel into a graduated test tube (Fig. 1).

Four animals were used for each determination so as to minimize possible error from individual animal variation. The animals were weighed and hydrated by introducing

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*Trainee, National Cancer Institute.

water, amounting to 5 per cent of their body weight, into their stomach by a No. 8 plastic infant feeding tube. Two animals were placed in each cage and the urine was collected and measured at 15-minute intervals for a three-hour period. Following an hour of rest,

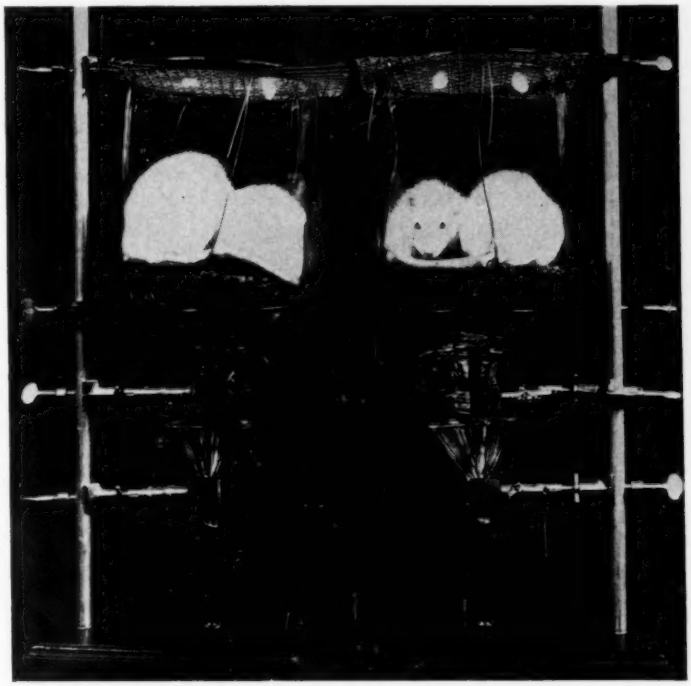

Fig. 1.

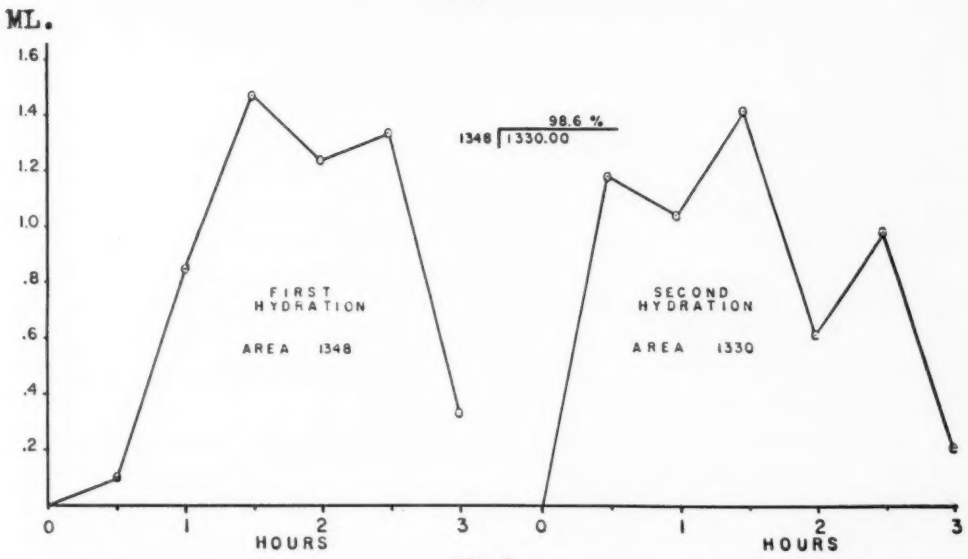

Fig. 2.

the same animals were rehydrated in the same manner and the output again measured for a three-hour period. The urinary output was plotted against time on arithmetic millimeter (Dietzgen) paper (Fig. 2). The area beneath these two curves was measured with

a Keuffer-Esser planimeter. Therefore, the volume-time relationship could be compared rather than the volume alone measured. It was found that the total output for each three-hour period was approximately 90 per cent of the amount ingested and the output following the second hydration was equal to the first. Thus, the first curve could be used as a control and the second for the determination of antidiuretic activity in the patient's plasma.

The above procedure was repeated using the same hydration technique, but at the time of the first hydration 2.5 ml. of water were injected subcutaneously along the dorsal spine, and at the time of the second hydration 2.5 ml. of the patient's plasma was injected into the same area. A comparison of the area beneath the output curves gives the degree of antidiuretic activity in the plasma. This is expressed as a percentage effect; i.e., the area beneath the second curve is a certain per cent of the area of the first.

Twenty milliliters of whole blood was drawn from the antecubital vein of the patient and heparinized with two drops of liquid heparin per 5 ml. of blood. The blood was centrifuged at 2,300 g for 30 minutes (International Centrifuge, Size 2, at 3,000 r.p.m.). The plasma was then removed and used unaltered.

RESULTS

Nonpregnant female patients between the ages of 15 and 40 were used to establish a control level. However, it was found that the normal individual under ordinary conditions and in water balance has a considerable amount of circulating antidiuretic activity. The average area beneath the second hydration curve was 50 per cent of the area beneath the first. Therefore, the antidiuretic activity of the plasma was approximately twice that of the water used in the first hydration. In order to establish a standard curve, it was necessary to remove as much of this intrinsic ADH as possible. This was done by superhydration of a group of 20 nonpregnant female patients. They were given fluids amounting to 3 per cent of their body weight. One-half of this volume was given intravenously and one-half orally, the entire volume being given during a one-hour period. Theoretically, this superhydration diluted the existing ADH and the hemodilution, through the osmoreceptors, reflexly arrested the secretion of ADH so that circulating antidiuretic activity was at a minimum. Blood was drawn before and after the superhydration, and ADH assays were made. The plasma before hydration produced a curve the area of which was 50 per cent of the control curve, but after the hydration procedure the output curve area was 88 per cent of the control.

It will be noted that the second curve, following the injection of water, was 100 per cent and that following the injection of the hydrated plasma it was 88 per cent. The difference perhaps represented other substances in the plasma, the ADH activity being at an irreducible minimum by this procedure and theoretically equaling 100 per cent.

To establish a standard curve of known amounts of Pitressin, dilutions of Pitressin were made using the hydrated plasma. As the amount of Pitressin was increased, the per cent area decreased. Plasma from forty nonpregnant, superhydrated female patients was used with different dilutions of Pitressin. Hydrated plasma with 0.0005 unit of Pitressin per 100 grams of rat gave an area of 86 per cent, or the same as the hydrated plasma alone. Plasma with 0.001 unit per 100 grams of rat gave 58 per cent, and 0.1 unit per 100 grams

gave 23 per cent. Adding more Pitressin, to as much as one unit per 100 grams, did not produce more effect. The maximum antidiuretic activity was between 20 and 30 per cent and this was produced by 0.1 unit per 100 grams (Fig. 3).

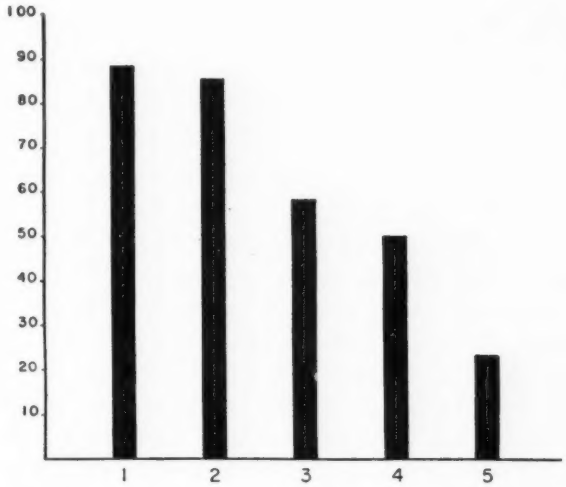

Fig. 3.—(1) Plasma from superhydrated patients; (2) 0.0005 unit of Pitressin per 100 Gm.; (3) 0.001 unit of Pitressin per 100 Gm.; (4) plasma from nonhydrated patients; (5) 0.1 unit of Pitressin per 100 Gm.

SUMMARY

A method has been presented to establish by bio-assay the antidiuretic hormone levels in plasma. A standard was prepared using serial dilutions of Pitressin in hydrated plasma thereby allowing comparison of the response in patients with unknown degrees of antidiuretic activity.

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SIMULTANEOUS MEASUREMENT OF GLUCOSE AND FRUCTOSE

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DURING a study of intermediary carbohydrate metabolism, it seemed advantageous to observe the metabolism of fructose in the presence of glucose and determine the levels of both compounds. The use of anthrone reagent for this purpose has been suggested by several authors. An ultramicromethod was developed by Bonting[1] and a macromethod by Mokrasch[2]; however, he did not apply it to biologic samples. Since the anthrone method was used routinely in this laboratory for the determination of glucose and the simultaneous determination of sucrose and inulin,[3] a suitable procedure which used a modified anthrone reagent was developed for simultaneous determination of glucose and fructose based on controlled heating for a constant time at two temperatures.

MATERIALS AND METHODS

Reagents.—1. Anthrone reagent: 500 ml. of concentrated sulfuric acid (Baker's analyzed) was added to 250 ml. of distilled water and cooled to room temperature. Two Gm. of anthrone (Jasonol's Chem. Corp.) was dissolved in 250 ml. concentrated H_2SO_4 and added to the dilute acid. During the mixing, the flask was kept in a cool water bath to prevent overheating which will destroy the reagent.

2. Stock standard glucose solution: 100 mg. of glucose was dissolved in and diluted to 100 ml. with 0.25 per cent benzoic acid solution.

3. Stock standard fructose solution: 100 mg. of fructose was dissolved in and diluted to 100 ml. with 0.25 per cent benzoic acid solution.

4. Working standards: 60 μg per milliliter was prepared by diluting 3 ml. of each of the stock standards to 50 ml. with distilled water. These working standards were prepared fresh every day.

5. 10 per cent zinc sulfate.

6. 0.5 N sodium hydroxide.

Procedure.—Proteins were precipitated by Somogyi's method[4]: 0.25 ml. of plasma was transferred with a micropipette to a 13 × 100 mm. test tube containing 5 ml. distilled water; 1.0 ml. 10 per cent $ZnSO_4$ was added, mixed, and allowed to stand five minutes. After this interval, 1.0 ml. of 0.5 N NaOH was added, and the tube was capped with parafilm, shaken vigorously, and centrifuged.

Two 1.0 ml. aliquots of each supernatant were placed in 19 × 150 mm. Coleman cuvettes. For the reagent blanks, 1.0 ml. of distilled water was used in place of the sample. Duplicate standards of glucose and of fructose were included in each run. Each cuvette was immersed in a beaker of cold water and shaken during the addition from a burette of 10 ml. of 0.2 per cent anthrone reagent. To prevent erratic color development due to heat of mixing,

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the samples having been thoroughly mixed were placed in a cold water bath. One of the two sets of samples was then placed in a 75° C. water bath and the other in a 100° C. water bath. After heating for seven minutes, the tubes were removed and cooled in running water for 10 minutes. The optical densities were read in a Coleman Junior spectrophotometer at 630 mμ. The spectrophotometer was adjusted to read zero density through the appropriate reagent blank. Optical densities of each sample at 75° C. and 100° C. were recorded.

Calculations.—The concentrations of glucose and fructose are calculated by substitution in the following equations:*

$$\text{Glucose conc/ml.} = \frac{(\text{O.D.U.}_{100} \times \text{KF}_{75}) - (\text{O.D.U.}_{75} \times \text{KF}_{100})}{(\text{KF}_{75} \times \text{KG}_{100}) - (\text{KF}_{100} \times \text{KG}_{75})}$$

$$\text{Fructose conc/ml.} = \frac{(\text{O.D.U.}_{75} \times \text{KG}_{100}) - (\text{O.D.U.}_{100} \times \text{KG}_{75})}{(\text{KF}_{75} \times \text{KG}_{100}) - (\text{KF}_{100} \times \text{KG}_{75})}$$

These calculations are derived from solving the following equations simultaneously:

$$(\text{KG}_{75} \times \text{conc G}) + (\text{KF}_{75} \times \text{conc F}) = \text{O.D.U.}_{75}$$

$$(\text{KG}_{100} \times \text{conc G}) + (\text{KF}_{100} \times \text{conc F}) = \text{O.D.U.}_{100}$$

when: O.D.G. = optical density of glucose standard,
O.D.F. = optical density of fructose standard,
O.D.U. = optical density of unknown mixture,

$$\text{KG}_{75} = \frac{\text{O.D.G. at 75°}}{\text{conc glucose/ml.}},$$

$$\text{KG}_{100} = \frac{\text{O.D.G. at 100°}}{\text{conc glucose/ml.}},$$

$$\text{KF}_{75} = \frac{\text{O.D.F. at 75°}}{\text{conc fructose/ml.}},$$

$$\text{KF}_{100} = \frac{\text{O.D.F. at 100°}}{\text{conc fructose/ml.}}.$$

RESULTS

Fig. 1 shows the color development curves of glucose and fructose at time intervals of 1 to 12 minutes under the conditions of the test. These curves agree essentially with those given by Koehler.[5] Although fructose reaches its maximum color development in 2 to 3 minutes at these temperatures and begins to decrease, the decrease is gradual. Reproducibility was poor when the heating time was 5 minutes; recovery values were high at 10 minutes' heating time although good reproducibility was obtained. The slopes of segments of the curves were calculated. For the segment from 6 to 8 minutes, the slopes of the curves of both compounds at both temperatures were found to be minimal. Since this was at a point on the curve near maximum color development, 7 minutes was chosen as the heating time. Errors from small variations in heating were negligible.

Seven minutes' heating time gave recoveries for fructose averaging 102.1 ± 2.1 per cent, for glucose 100.4 ± 1.1 per cent, and for total sugar 101.1 ± 0.7 per cent from mixtures of standard solutions (Table I). Recovery of fructose added to plasma was 98.9 ± 2.1 per cent (Table II). Plasma used for these recoveries consisted of four separate pools, each consisting of either 10 or 12

*These calculations have been facilitated in this laboratory by using a Monroe Calculator. By setting the multiplication up on the left-hand side of the keyboard, using the clear multiplier for the first multiplication and the negative multiplier for the second, the remainder is left in position to be divided without re-entering the numerator on the keyboard. The divisor is constant for both equations.

TABLE I. RECOVERIES OF GLUCOSE AND FRUCTOSE FROM MIXTURES OF STANDARD SOLUTIONS

FRUCTOSE			GLUCOSE			TOTAL SUGAR		
MICROGRAM/ML. ADDED	MICROGRAM/ML. MEASURED	PER CENT RECOVERY	MICROGRAM/ML. ADDED	MICROGRAM/ML. MEASURED	PER CENT RECOVERY	MICROGRAM/ML. ADDED	MICROGRAM/ML. MEASURED	PER CENT RECOVERY
40	40.6 ± 0.2	101.5	20	20.3 ± 0.5	101.5	60	60.9 ± 0.6	101.5
30	30.4 ± 0.4	101.3	30	29.8 ± 0.6	99.3	60	60.2 ± 0.7	100.3
20	20.7 ± 0.3	103.5	40	40.2 ± 0.5	100.5	60	60.9 ± 0.5	101.5

TABLE II. RECOVERY OF FRUCTOSE FROM PLASMA

NUMBER OF SAMPLE	GLUCOSE (MG. %)	FRUCTOSE ADDED (MG. %)	FRUCTOSE MEASURED (MG. %)	RECOVERY OF ADDED FRUCTOSE (PER CENT)
1	95.7 ± 2.30	0	2.2 ± 0.10	
		50	51.1 ± 1.38	97.9
		100	101.7 ± 1.65	99.5
2	142.5 ± 1.05	0	2.7 ± 0.03	
		50	51.8 ± 1.21	98.3
		75	75.0 ± 0.50	96.5
3	97.7 ± 1.16	0	1.4 ± 0.02	
		50	50.2 ± 1.17	97.7
		100	99.1 ± 0.78	97.7
4	98.3 ± 2.42	0	1.8 ± 0.10	
		50	53.4 ± 0.53	103.1
		75	77.2 ± 0.72	100.5

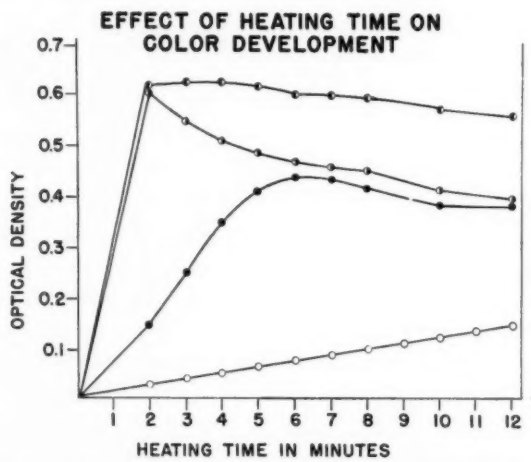

Fig. 1.—Effect of heating time on color development of glucose and fructose with anthrone reagent: Glucose at 75° [○]; Fructose at 75° [◐]; Glucose at 100° [●]; Fructose at 100° [◑]. 1 ml. of standard solution containing 60 μg of the hexose was heated with anthrone reagent at 75° and 100°.

samples. When determinations were made on these plasma samples before addition of fructose, values for fructose of 2.2, 2.7, 1.4, and 1.8 mg. per cent were obtained. These values were considered in calculating recoveries.

Fig. 2 shows the standard curves of solutions of glucose and fructose. Linearity is maintained with glucose concentrations from 10 to 80 μg per milliliter and fructose concentrations from 10 to 60 μg per milliliter.

With mixtures of equal parts of glucose and fructose, levels up to a total sugar content of 60 to 80 μg per milliliter may be accurately determined (Fig.

3). A comparison of these curves shows that the optical densities are additive. There is good agreement between the theoretical optical density expected and that actually obtained with the mixture. Best results were obtained if the optical density of the mixture did not exceed 0.6. Above this point the slope began to decrease slightly and eventually leveled off. By varying the original dilution, concentrations were kept in this range of best precision.

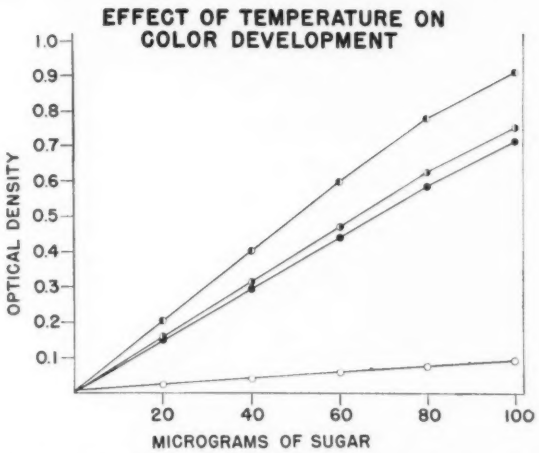

Fig. 2.—Effect of temperature on color development of glucose and fructose with anthrone reagent: Glucose at 75° [○]; Fructose at 75° [◐]; Glucose at 100° [●]; and Fructose at 100° [◑]. Standard solutions heated 7 minutes with anthrone reagent at 75° and 100°.

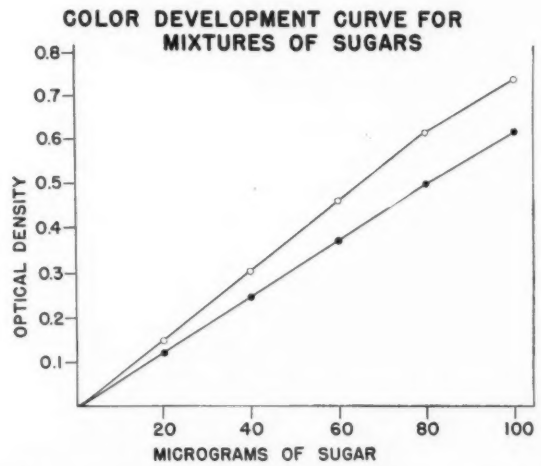

Fig. 3.—Color development curve for mixture of sugars. Standard solutions containing equal proportions of glucose and fructose were heated for 7 minutes with anthrone reagent at 75° [●], and 100° [○].

Since anthrone changes color slightly upon heating, the reagent blank as previously described must always be prepared and used as the zero reference. This maintains the intercept of the standard curve at zero and obviates individual corrections for the samples. Under these conditions only one concentration of each standard need be run with each series of samples. Since constants were used in the calculations, any error in the standards will be reflected in all the

tests; therefore, duplicate standards were always run. Good agreement was found between duplicates (± 0.003 O.D.). Day-to-day variations in the slopes of standard curves were small. These variations and those due to reagents were eliminated by using blanks and standards prepared with each series.

DISCUSSION

The anthrone reagent (0.05 per cent with thiourea) described by Roe[6] lacked sufficient sensitivity for use in this study, i.e., 60 micrograms of glucose at 100° C. gave an optical density of 0.2 vs. 0.44 with our reagent. Scott[7] showed that the anthrone concentration should be greater than 0.18 per cent for optimum color development. A reagent with 0.2 per cent anthrone concentration was selected because it had good sensitivity. It proved to be stable for several weeks if stored in a dark bottle. Recrystallization of the anthrone used here was found to be unnecessary.

Calculations based on solution of simultaneous equations[2] gave a truer representation of the concentration of the constituents than those based on the subtraction of optical densities.[1] In the latter case, results would be biased in favor of one of the constituents.

SUMMARY

A method is presented for simultaneous determination of glucose and fructose in biologic materials suitable for use in studies of intermediary carbohydrate metabolism. The method is based on the development of color with an anthrone reagent at 75° and 100° C. Concentrations were calculated by solving simultaneous equations.

Good precision was obtained. The coefficients of variation for determination in mixtures of standard solutions were ± 1.1 per cent for glucose and ± 1.5 per cent for fructose (54 determinations). In plasma the coefficients of variations were ± 1.7 per cent for glucose and ± 1.5 per cent for fructose (36 determinations).

Recoveries from mixtures of standard solutions averaged 100.4 per cent for glucose and 102.1 per cent for fructose. Recoveries of fructose from plasma averaged 98.9 per cent.

Curves showing the effect of time and temperature on the reaction are given.

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RESULTS OF A MODIFIED RHEUMATOID HEMAGGLUTINATION TEST; CORRELATION OF RESULTS WITH OCCURRENCE OF C-REACTIVE PROTEIN

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INTRODUCTION

RECENT reports[1-3] have adequately reviewed the origins and uses of the rheumatoid hemagglutination test. The procedure described here utilizes the rapid mineral acid dilution method of Neurath and co-workers[4] for separation of the euglobulin fraction which then is used as in Ziff and associates' agglutination method.[1] The modification offers simplification of method, without loss of specificity, in the following respects: (1) In contrast to methods employing whole serum, the sheep cell absorption step can be omitted.* The present method of euglobulin precipitation requires less time and material (sheep cells) than does the absorption step. (2) In contrast to previously reported methods employing euglobulin, the delays attendant on separation of the euglobulin fraction by dialysis are greatly reduced. The results obtained, using the method described, compare favorably with those published and reviewed by others.[1-3] They are correlated in the present study with the occurrence of an acute phase reactant (C-reactive protein).

METHODS

Patients.—Patients were divided into groups as follows: In Group I were patients with clinically unequivocal rheumatoid arthritis, without regard to stage or grade of activity. In Group II were all other patients, the following subgroups being recognized: In Group IIA were patients with suspected but not clinically definite rheumatoid arthritis and patients with known or strongly suspected other "collagen" disease, excluding rheumatic fever. In Group IIB were all other patients with joint symptoms, including rheumatic fever. In Group IIC were patients with a variety of disorders but no evident joint involvement.

C-Reactive Protein Tests.—C-reactive protein tests were done on most of the patients in Groups I, IIA, and IIB, but not on most of the patients in Group IIC. Various lots of C.R.P. Antiserum Schieffelin were used. Tests were read as 0 to 4+ according to the number of millimeters of precipitate formed in a capillary tube of uniform size.

Hemagglutination Tests.—Hemagglutination tests were done as follows:

(a) *Mineral acid:* 0.0027N hydrochloric acid was prepared fresh daily by diluting 2.7 ml. of a stock solution of N/10 hydrochloric acid to 100 ml. with distilled water.

(b) *Preparation of the euglobulin fraction:* Test sera were inactivated by heating at 56° C. for one-half hour. In a 100 ml. cellulose nitrate tube, one volume of clear serum

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*Heterophile agglutinins appeared in the euglobulin fraction in one of over 400 sera tested; when this rare occurrence presented itself, preliminary absorption of serum with sheep cells became necessary for that serum. Appropriate controls were included routinely to detect heterophile agglutination.

was diluted by the slow addition (with continuous rotation of the tube) of 9 volumes of 0.0027N hydrochloric acid from a 50 ml. burette. A pH within the range of 6.1 to 6.4 was thereby attained. It was not necessary to measure the pH of the specimen other than as a check on the 0.0027N hydrochloric acid. The tube was immediately stoppered with a rubber stopper, placed in an ice bath, and refrigerated at 4 to 8° C. for 30 to 60 minutes. The euglobulin precipitate then was centrifuged in the cold in a small angle-head Servall centrifuge at 3,000 r.p.m. for 10 minutes. The clear supernate was discarded by decanting from the firmly packed pellet, and last traces of the supernate were removed either by draining the tube inverted over filter paper or by wiping the inner wall thoroughly with a gauze sponge. The precipitate was not washed. The concentrated euglobulin was then dissolved in an added 0.5 ml. of 0.9 per cent NaCl solution, stirring with a glass rod if necessary.* The euglobulin was transferred quantitatively to a 15 ml. graduated Pyrex centrifuge tube, using 2 additional 0.5 ml. portions of saline for washing the original tube, and was then made up to original serum volume (usually 2.0 ml.) with saline. Any traces of insoluble material were removed by centrifugation in an International No. 1 centrifuge at 2,000 r.p.m. for 10 minutes at room temperature. Then the supernate containing the euglobulin was decanted off, ready for use in the hemagglutination test. It was usually colorless to slightly opalescent. Chylous or markedly hemolyzed serum was avoided when possible.

(c) *Sheep erythrocytes:* Whole blood was collected aseptically from sheep into equal volumes of Alsever's Solution (modified)[5] and stored in 60 ml. bottles at 4 to 8° C. for future use up to two months.

(d) *3 per cent sheep cell suspension:* The required volume of blood for the day's test was withdrawn aseptically from a 60 ml. bottle and washed 3 times with 6 volumes of 0.9 per cent NaCl solution in a 15 ml. graduated centrifuge tube, using an International No. 1 centrifuge at 1,800 r.p.m. for 5 minutes and a final 2,000 r.p.m. for 10 minutes. The packed cells were diluted to a 3 per cent suspension with 0.9 per cent NaCl solution.

(e) *Rabbit antisheep cell serum (amboceptor):*† Rabbit antisheep cell serum was kept as a 1:100 amboceptor solution, prepared as follows and stored at 4 to 8° C.:

0.9 per cent NaCl solution	94.0 ml.
Phenol (5 per cent in 0.9 per cent NaCl)	4.0 ml.
Glycerinized amboceptor	2.0 ml.

(f) *Determination of the basic agglutinating titer of amboceptor:* Amboceptor dilutions were prepared as follows:

Dilutions	1:100	1:133.3	1:200	1:266.6	1:400	1:533.3	1:800	1:1,066
Amboceptor	0.8	0.6	0.4	0.3	0.2	0.15	0.1	0.075
0.9 per cent NaCl	0.0	0.2	0.4	0.5	0.6	0.65	0.7	0.725

The titration then was carried out by mixing 0.5 ml. of each dilution with 1.0 ml. of 0.9 per cent NaCl and 0.5 ml. of 3 per cent sheep cell suspension. Each tube of the titration was shaken thoroughly and refrigerated at 4 to 8.° C. for 15 to 18 hours. The titration was run on the day preceding that of the test run. The end point agglutinating titer was determined by holding each of the tubes in front of a good light source and agitating the tube with the forefinger until all sedimented erythrocytes were well suspended; the degree of clumping was noted and graded as 0 to 4+. The highest dilution showing a 2+ agglutination was termed the basic agglutinating titer. Titer was expressed as the reciprocal of the initial dilution before the addition of the other reagents.

(g) *Sensitization of sheep cells:* Equal volumes of 3 per cent sheep cell suspension and appropriately diluted antisheep cell amboceptor (one-half of the basic agglutinating titer; e.g., with B.A.T. 200, diluted 1:400) were mixed thoroughly and let stand at room temperature for a minimum of 10 minutes before use.

*Solution of the euglobulin was facilitated by rotating the tube for a few seconds under a warm tap (at approximately 50° C.).

†Obtained from Swanson Biochemical Laboratories, San Antonio, Tex.

(h) *Titration of hemagglutinins:* Serial twofold dilutions beginning with 1:7 and carried through 1:896 were prepared by addition as follows: In a series of 9 tubes, the following quantities of 0.9 per cent NaCl solution: first tube, 1.8 ml.; second through eighth tubes, 0.5 ml.; ninth tube, 0.25 ml. To the first tube, 0.3 ml. of euglobulin solution (1:7); 0.5 ml. mixed and transferred to each of Tube 2 (1:14) and Tube 9 (control); 0.6 ml. discarded from Tube 1; 0.5 ml. transferred serially from Tube 2 through Tube 8 (1:896); 0.5 ml. discarded from Tube 8. Ont-half milliliter of sensitized sheep cells to each of Tubes 1 through 8 and to a tenth tube containing 0.5 ml. of 0.9 per cent NaCl solution (control for spontaneous agglutination of sensitized cells). To Tube 9, 0.25 ml. of 3 per cent sheep cell suspension (nonsensitized; control for heterophile agglutination). All tubes were shaken vigorously and refrigerated at 4 to 8° C. for 15 to 18 hours. The test was read immediately upon removal from the refrigerator, as follows: Each tube was shaken enough to dislodge the well-formed button of cells in the bottom of the tube. The 2 to 4+ agglutinated cells were more easily dislodged than those in the trace to 1+ and negative tubes, and the cells settled out again much more rapidly than in the weakly agglutinated and negative tubes. The highest dilution exhibiting 2+ agglutination (approximating and not exceeding the degree of agglutination which was selected as the end point in determining the basic agglutinating titer of the amboceptor) was termed the agglutinating titer of the euglobulin and was expressed as the reciprocal of the initial dilution before the addition of sensitized sheep cells.

RESULTS

Results are summarized in Table I and illustrated graphically in Fig. 1. Titers up to 14 were frequently encountered in individuals with no joint dis-

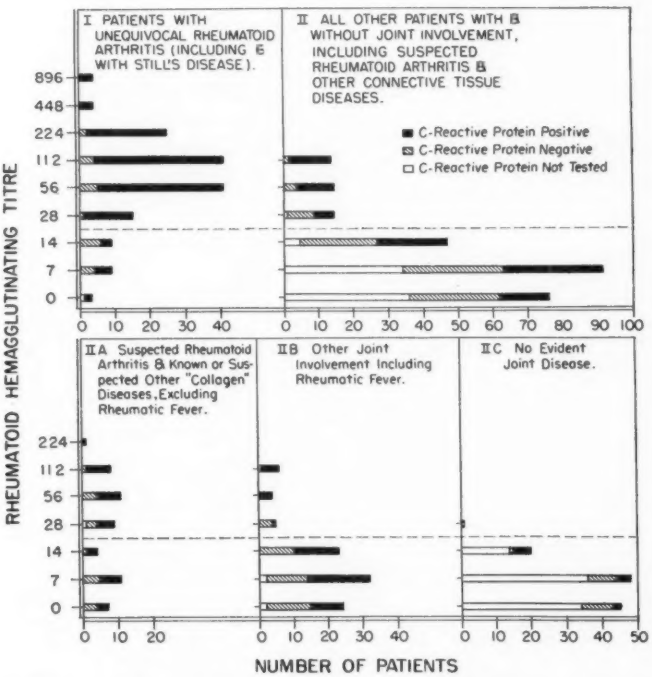

Fig. 1.—Distribution of hemagglutination titers and C-reactive protein reaction in all groups of patients.

ease. A sharp dividing line was encountered at that point above which the 114 patients of Group IIC with no joint involvement did not go, with one

TABLE I. SUMMARY OF HEMAGGLUTINATION AND C-REACTIVE PROTEIN DATA ON ALL PATIENTS

DIAGNOSIS*	TOTAL NUMBER OF PATIENTS	R.H.A. TITER 1:28 OR >	CRP+/NUMBER TESTED	R.H.A. TITER 1:14 OR <	C.R.P.+/ NUMBER TESTED
I. Unequivocal R. A. (including 6 Still's)	151	*130* (86%)	118/130 (91%)	*21* (14%)	10/20 (50%)
II. All others	258	*45* (17%)		*213* (83%)	
A. Known or probable other "collagen" disease	51	*29* (57%)	21/28 (75%)	*22* (43%)	12/22 (55%)
(1) Early R. A., suspected	13	7	4/6	6	3/6
(2) Still's, suspected	1	0		1	1/1
(3) Sjogren's, suspected	1	0		1	1/1
(4) S.L.E. known	9	4	3/4	5	2/5
S.L.E. suspected	5	3	2/3	2	1/2
(5) Scleroderma, known	12	6	5/6	6	4/6
(6) P.N., known	3	3	3/3	0	
P.N., suspected	4	4	2/4	0	
Polyangiitis	2	1	1/1	1	0/1
(7) D.M., suspected	1	1	1/1	0	
B. Other joint involvement	93	*15* (16%)	10/15 (67%)	*78* (84%)	38/73 (52%)
(1) Palindromic	1	0		1	1/1
Palindromic, suspected	1	0		1	1/1
(2) Rheumatoid spondylitis	13	2	2/2	11	7/10
(3) Ulc. colitis with arthritis	4	3	3/3	1	1/1
(4) G.C. arthritis, known	1	0		1	0/1
G.C. vs. R.A.	2	1	1/1	1	1/1
(5) Sarcoidosis with joint involvement	1	0		1	1/1
(6) Acute, R.F., known	8	1	1/1	7	7/7
Acute R.F., suspected	2	0		2	2/2
(7) Drug reactions	6	1	1/1	5	3/5
(8) Reiter's, suspected	1	0		1	1/1
(9) Gout	2	0		2	1/2
(10) Degenerative arthritis	30	3	0/3	27	5/24
(11) Charcot joint	1	0		1	0/1
(12) Hemophilia with arthralgia	1	0		1	0/1
(13) Periarticular disease	12	3	1/3	9	2/8
(14) Shoulder hand	4	0		4	3/4
(15) Pulm. osteoarthropathy secondary to Ca. of bronchus	1	0		1	1/1
(16) Metastatic bone tumor secondary to Ca. of breast	1	0		1	1/1
(17) Fibrosarcoma of femur	1	1	1/1	0	
C. No evident joint disease	114	*1* (1%)	0/1	*113* (99%)	11/38

*R.A. = rheumatoid arthritis; S.L.E. = systemic lupus erythematosus; P.N. = periarteritis nodosa; D.M. = dermatomyositis; R.F. = rheumatic fever.

exception (a patient with history suggestive of chronic urticaria which was never observed). This is interpreted as indicating that false-positive tests for technical reasons were infrequent.

Table II summarizes the correlative data relating hemagglutination titers to the degree of reaction of serum with C-reactive protein antiserum. It is to be emphasized that the estimation of C-reactive protein by measuring the height of the column of precipitate formed in a capillary tube of uniform size must be regarded as only roughly quantitative and can be useful only to indicate possible worth-while directions for later investigation employing more exact techniques. On the other hand, as a means of detecting the presence or absence of C-reactive protein in the serum, the capillary method is highly reliable, and the over-all percentage of reacting sera recorded in Table I can be accepted without reservation.

TABLE II. CORRELATION OF EUGLOBULIN HEMAGGLUTINATING TITER WITH THE DEGREE OF REACTION OF WHOLE SERUM WITH C-REACTIVE PROTEIN ANTISERUM*

GROUP**	NUMBER OF PATIENTS	PER CENT WITH + C.R.P.	AVE. HA TITER	AVE. + C.R.P.	CORRELATION COEFFICIENT	SIGNIFICANCE
I	150	85%	123	3.1	0.2216	High ($p = <0.01$)
IIA	50	66%	42	2.1	0.1727	None ($p = >0.05$)
IIB	88	55%	18	1.9	0.1982	None ($p = >0.05$)

*See Results.

**I = Clinically unequivocal rheumatoid arthritis.

IIA = Suspected rheumatoid arthritis; known and suspected other "collagen" disease, excluding rheumatic fever.

IIB = All other patients with joint involvement, including rheumatic fever.

DISCUSSION

The technique described here for the rheumatoid hemagglutination test is one which can be done with meticulous care in any good serologic laboratory by well-trained technicians. Each laboratory must gain its own experience in reading the tests and establish its own base line observations for significance of titer. With this background, the test furnishes certain useful information when a significantly high titer is encountered. As with most laboratory tests, the information must be fitted into the over-all clinical picture. In our experience, an elevated titer (28 or higher) is not diagnostic of any single disease entity. It is nonetheless encountered with sufficient rarity in diseases unrelated to the so-called "collagen" group that it can, on occasion, be a significant observation added to other evidence pointing in the direction of connective tissue disease.

Thus far there are few correlative studies reported on the relation of rheumatoid hemagglutinating factor to acute phase reactants in serum. Recently Shetlar and associates[6] have noted no significant correlation between the presence of C-reactive protein and an elevated hemagglutinating titer in the serum of patients with rheumatoid arthritis. In the present series of patients with rheumatoid arthritis whose sera were tested also for the presence of C-reactive protein, C-reactive protein could be demonstrated in 91 per cent of the high-titer hemagglutinating group and in 50 per cent of the low-titer group. There

was a highly significant over-all correlation between hemagglutinating titer and degree of C-reactive protein precipitation (as roughly quantitated by capillary tube method; see Results). Such correlation did not occur in the other groups of patients tested. The possible significance of this observation must await additional and more exact quantitative appraisals of acute phase reactants in sera subjected to hemagglutination tests. It would seem from the present preliminary scanning that such appraisal in additional clinics might be worth while.

SUMMARY

1. A modified technique for the performance of the rheumatoid hemagglutination test is described, using a euglobulin fraction precipitated from serum by Neurath and associates'[4] method of mineral acid dilution.

2. Significantly elevated hemagglutinating titers were obtained in 86 per cent of 151 patients with clinically unequivocal rheumatoid arthritis, in 57 per cent of 51 patients with known or suspected other "collagen" disease or suspected early rheumatoid arthritis, in 16 per cent of 93 patients with other joint involvement including rheumatic fever, and in one of 114 patients with no evident joint disease.

3. C-reactive protein was present in 86 per cent of patients with clinically unequivocal rheumatoid arthritis—in 91 per cent of those with elevated hemagglutinating titers, and in 50 per cent of those with low-titer or absent hemagglutination.

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THE OPTOCHIN SENSITIVITY TEST: A RELIABLE METHOD FOR IDENTIFICATION OF PNEUMOCOCCI

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ALTHOUGH the ability of Optochin (hydrochloride of ethylhydrocupreine) to inhibit the growth of the pneumococcus has been known for many years,[1, 2] it is only recently that the suggestion has been made that this specific sensitivity might be used for the laboratory identification of pneumococci. In 1955, Bowers and Jefferies[3] described a simple procedure employing paper disks saturated with Optochin on agar plates. These workers reported that growth of bile-soluble strains of *Streptococcus pneumoniae* was inhibited uniformly by this drug, whereas bile-insoluble strains of alpha hemolytic streptococci almost always were resistant. These results led them to suggest that Optochin sensitivity might replace the time-consuming and often equivocal bile-solubility method for identifying pneumococci in the clinical bacteriology laboratory. None of the strains of pneumococcus used by Bowers and Jefferies had been identified by serologic typing.

It is the purpose of this communication to describe the results obtained with the routine use of the Optochin sensitivity test in a clinical bacteriology laboratory and to correlate the findings with serologic typing of pneumococci and the results of bile-solubility tests.

METHODS

During the early part of the study, filter paper disks (Schleicher and Schuell #740-E, 6.35 mm. in diameter) were immersed in a 1:4,000 solution of Optochin in distilled water and placed immediately upon the surface of seeded blood agar plates as in the usual disk sensitivity test. Preliminary tests had shown that the 1:4,000 concentration of Optochin was otpimal for differentiating pneumococci. In the latter part of the study, commercially produced dehydrated disks (Baltimore Biological Laboratory) were substituted for the wet disks. Both the wet and dry disks were tested in parallel against a series of 14 different serologic types of pneumococcus and eight different strains of alpha streptococcus. The results were identical. The wet disks, however, produced zones of inhibition of the sensitive strains 2 to 3 mm. greater in diameter than those around the dehydrated disks.

Organisms to be tested were streaked on plates of trypticase-soy agar containing six per cent human blood, Optochin disks were placed on the surface of the inoculated plates, and growth was examined after incubation for 18 hours at 37° C.

A total of 65 strains of pneumococcus representing 20 serologic types was tested. Fifty-one of these were tested immediately after isolation from the sputum of patients

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with pneumonia; 14 had been maintained as stock cultures for several months before testing. All were soluble in 10 per cent sodium desoxycholate.

A total of 72 strains of alpha hemolytic streptococcus was tested. Of these, 64 were freshly isolated and 8 had been maintained by repeated subculture for several months. All were shown to be insoluble when 10 per cent sodium desoxycholate was added to serum-broth cultures.

RESULTS

The growth of all 65 pneumococcal strains was inhibited by the Optochin disks. The zones of inhibition were uniform with clear-cut margins and their diameters varied from 15 to 30 mm. There was no correlation between the size of the zones of inhibition of growth and the serologic types of the pneumococci tested. However, the degree of inhibition clearly was related to the test inoculum in that the zones on plates showing heavy growth were always slightly smaller than those on plates showing comparatively light growth. The disks, however, were surrounded by a sharply demarcated area of complete inhibition of growth, regardless of the inoculum or the size of the zone.

Of the 72 strains of bile-insoluble alpha hemolytic streptococcus tested, 68 were unaffected by the Optochin disk. Four strains, however, showed narrow zones of partial or complete inhibition around the disks, 9 to 12 mm. in diameter. With all four organisms, growth on the rest of the plate was scanty. Therefore, these streptococci were again tested using a light and a heavy inoculum for each strain. The heavy inoculum was not inhibited at all although indistinct zones measuring 9 to 11 mm. in diameter again surrounded the disks on the plates showing light growth. These would not be confused with the wider, sharply defined zones of inhibition characteristic of pneumococci exposed to Optochin.

SUMMARY AND CONCLUSION

These sensitivity tests using Optochin disks with 65 strains of pneumococcus, representing 20 serologic types, and 72 strains of bile-insoluble alpha hemolytic streptococcus indicate a uniform sensitivity of pneumococci and a uniform resistance of streptococci to this substance. There is no overlapping of results when heavy inocula are used on test plates.

Because specific pneumococcal antiserum is no longer generally available and because the Quellung reaction and bile or desoxycholate solubility tests are time consuming and often difficult to interpret, the Optochin disk test fills a definite need in the clinical bacteriology laboratory. With the availability of dehydrated disks from commercial sources, it has become a simple, rapid, accurate, and inexpensive procedure for the identification of pneumococci.

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A SIMPLE APPARATUS FOR EXERCISING PATIENTS IN THE SUPINE POSITION FOR CARDIOPULMONARY EVALUATION

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AN APPARATUS has been designed to permit the exercising of patients in the supine position while undergoing cardiac catheterization, renal function studies, electrocardiographic evaluation, and pulmonary function determinations. Although instruments used for this purpose may be obtained commercially,[1] their cost makes them unavailable to many laboratories. The exercising of patients in the upright position is not often technically feasible. Furthermore, considerable hemodynamic changes are caused by the assumption of the upright position alone. An apparatus which permits the exercising of patients in the supine position appears, therefore, most desirable; particularly if construction is simple and cost is minimal. The apparatus designed meets these requirements.

Its construction is primarily of wood with the exception of the aluminum swival joints of the foot pedals (Fig. 1). Its light weight and portability permits use on practically any table simply by clamping it into position. The clamps used to keep the exerciser in place are ordinary "C" clamps. The pedals into which the patient's feet are strapped pivot horizontally to compensate for the angle created by the extension of the extremity. Work consists of pushing against the known resistance of a rubber tubing. The latex tubing used in construction has an outside diameter of 10 mm. and an inside diameter of 4.5 mm. A piece of rubber tubing approximately 34 cm. in length is cut. A length of 17.8 cm. is measured off and marked toward the center leaving an excess of approximately 8 cm. on either end to permit fastening. The tubing is then stretched from one point on the pedal arm to the other on the base of the apparatus so that the two marks representing 17.8 cm. are now 36.8 cm. apart. This is done on both sides. If equal stretch is obtained on the two sides, the pedal arm will remain exactly in the center or zero position (Fig. 1).

The calibration of this exerciser is accomplished by placing the apparatus on its side so that the arm acts as a balance. Since the center metal rod does not move with the arm, a needle is placed through the rods diameter horizontal to the arm, indicating the zero position. In turn, 10, 20, and 30 pound weights are placed on one end of the arm hanging freely beneath the pedal and the distance the arm moves each time is marked off where the needle points. The procedure is then repeated on the other side.

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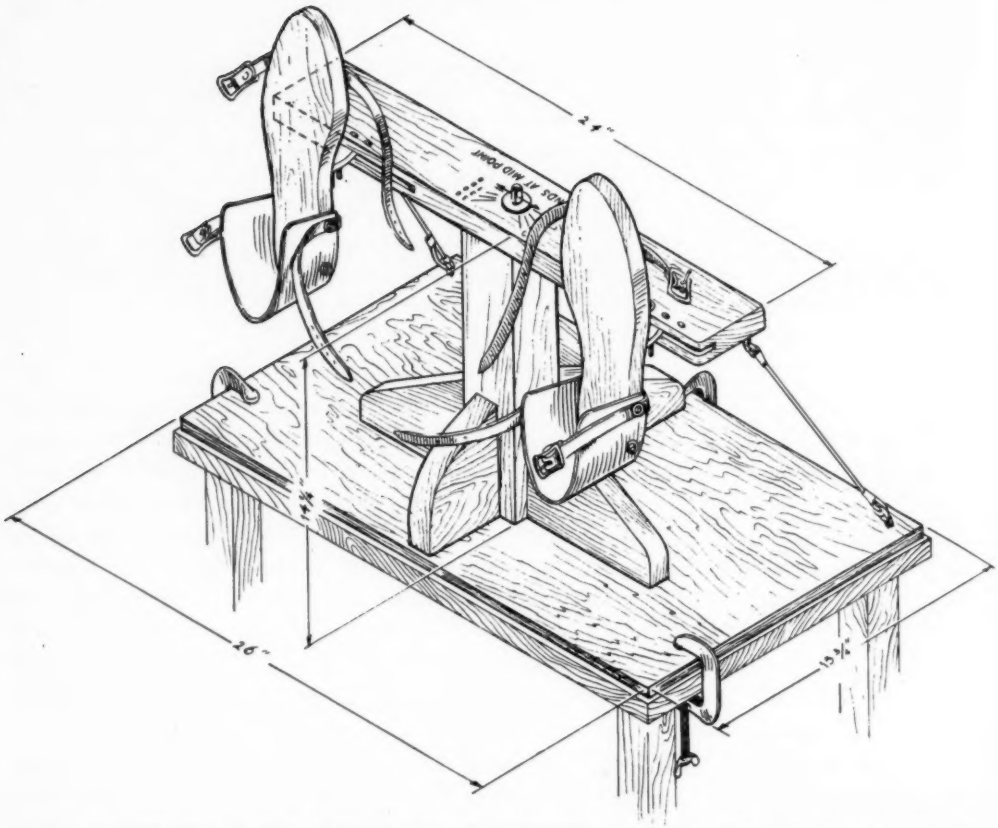

Fig. 1.—Drawing presenting dimensions and illustrating apparatus clamped into position on table.

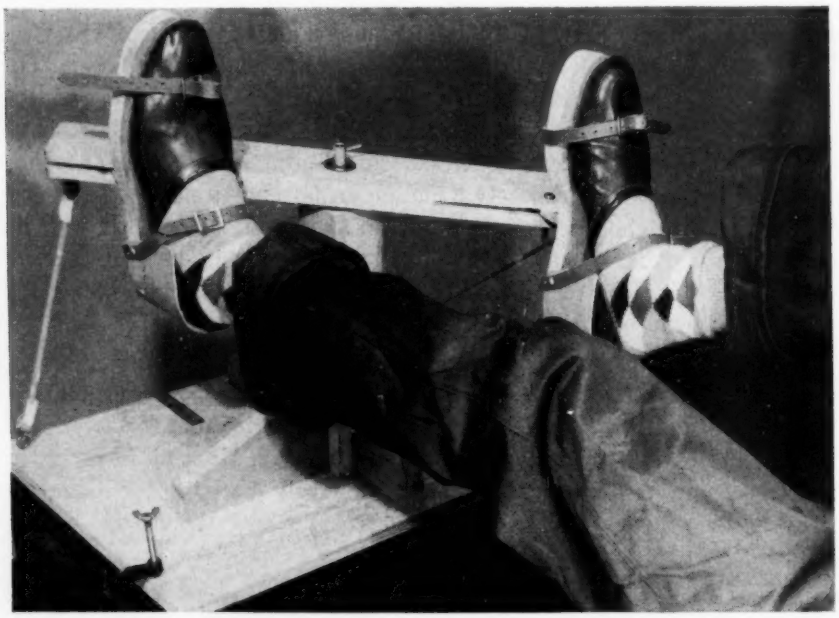

Fig. 2.—Photograph of exerciser in use.

Variation of the stroke distance required to obtain full extension of the extremities by movement of the apparatus on the table from a constant position of the patient permits a force range of zero to thirty pounds.

The foot pounds of work done per minute may be obtained by the formula:

$$W = (\tfrac{1}{2}F \times D) \text{ (strokes per minutes)}$$

W = work
D = distance rubber stretched from O position
F = force in pounds

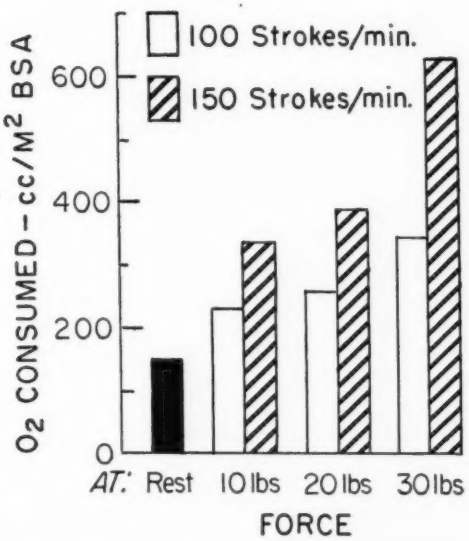

Fig. 3.—The effect of varying exercise on oxygen consumption. "Strokes" refers to the total number of strokes per minute.

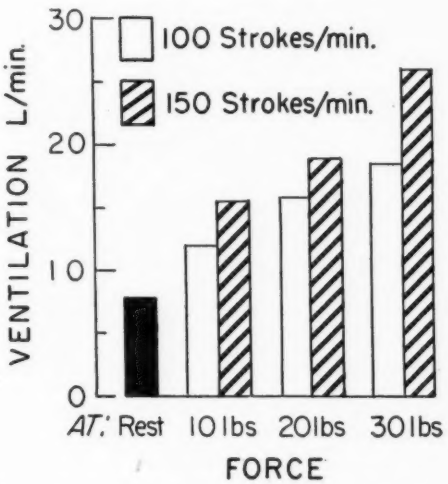

Fig. 4.—The effect of varying exercise on ventilation.

We have found that in the range 0 to 30 pounds each 10 pounds of added force produced an equal increment of length. A stroke of 10, 20, and 30 pounds

equaled a calculated work load of 1, 4, and 9 foot pounds per stroke, respectively. Approximately 1,500 foot pounds of work per minute could be accomplished on this apparatus.

Figs. 3 and 4 illustrate the mean oxygen consumption and ventilation of two normal subjects exercised with the apparatus. The average oxygen consumption and average ventilation were obtained after a steady state of exercise at each level of work had been reached. The steady state was reached in approximately nine minutes. A metronome was used as a pacemaker to regulate the number of strokes per minute. With work rates of 150 strokes per minute and equivalent loads of 30 pounds, oxygen consumption was increased approximately fourfold.

SUMMARY

A simple inexpensive apparatus for exercising patients in the supine position has been described.

Representative figures are included demonstrating the physiologic response which may be obtained when this apparatus is used.

We acknowledge the valuable criticism and advice of Dr. Rudolph E. Fremont, Chief of Cardiology Section. We are also deeply indebted to Robert Gilpatric of the Physical Medicine and Rehabilitation Service who aided in construction of this apparatus.

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A RAPID METHOD FOR RECOVERING SEROLOGICALLY ACTIVE GLOBULINS BY SODIUM SULFATE PRECIPITATION

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INTRODUCTION

A TECHNIQUE has been developed for the rapid recovery of serologically active globulins from serum samples of 1.0 ml. or less. The method which eliminates the necessity of a dialysis is a modification of the classic salting-out procedures employing sodium sulfate.[1-4] Globulins of high antibody activity and free of albumin can be obtained for serologic study in four hours.

MATERIALS AND METHODS

Sodium Sulfate Solutions.—The sodium sulfate solutions were prepared with reagent grade anhydrous sodium sulfate and double distilled water at 37° C. (water bath). The concentrations were so adjusted that the addition of 9.0 ml. of sodium sulfate solution to 1.0 ml. of serum resulted in the desired final concentration of salt. Solutions of 16.6, 20.0, 21.1, and 28.6 per cent sodium sulfate giving final concentrations on admixture with serum of 15.0, 18.0, 19.0, and 26.0 per cent were employed.

Fractionation Procedure.—To 1.0 ml. of the serum sample in a vial or centrifuge tube, was added 9.0 ml. of sodium sulfate solution. The mixing of the salt solution with the serum was carried out at 37° C. It was found that a floc which could be centrifuged most readily from suspension was obtained when the salt solution was added to the serum slowly and with constant agitation. After admixture the vials or centrifuge tubes were stoppered and incubated at 37° C. (water bath) for at least three hours, after which the samples were centrifuged. Centrifugation in a model SBV size 1 International centrifuge at 3,500 r.p.m. for 20 minutes was employed in these laboratories. The supernatant fluid containing albumin was removed with a capillary pipette attached to a vacuum source. One or two milliliters of double distilled water was then carefully layered over the precipitated globulin to dilute the residual layer of salt and albumin solution which had not been removed with the capillary pipette. The supernatant fluid was removed carefully as before. The precipitated globulin was dissolved in a minimal quantity of distilled water and the globulin solution was restored to the orginal serum volume (1.0 ml.) with additonal water.

Sera.—Representative rabbit and human serum samples containing antibodies to a variety of antigens were obtained through the cooperation of personnel of the Department of Bacteriology of The Ohio State University and the Laboratories of the Ohio State Department of Health. Each of the reconstituted globulin fractions was tested simultaneously with the untreated serum sample by standard serologic techniques.

Electrophoresis.—The various sera and serum fractions were analyzed by paper electrophoresis. Twenty cmm. of each sample was applied to Whatman filter paper (No. 3 mm.), previously wetted with Veronal buffer (pH 8.6; ionic strength 0.05). All supernatant

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fluids, i.e., those recovered after precipitation procedures, were dialyzed against distilled water and of necessity concentrated to 0.1 volumes by desiccation under vacuum to restore the concentrations of proteins to those present in the original serum sample. The treated paper was installed in a Durram-type tank (containing the Veronal buffer described above) and electrophoresis was conducted for five and one-half hours at a power potential of 150 to 200 volts per sheet of filter paper (33 × 22.9 cm.). After drying in a hot-air oven (160° C. for ten minutes), the test paper was cut into strips, representing each of the samples analyzed, stained by the bromphenol-blue method and examined.

Precipitin Reaction.—Precipitin tests were performed according to the classical ring procedure. A small quantity of the test material (reconstituted globulin or concentrated supernatant fluid) was layered over the appropriate antiserum (antihuman serum or antihuman albumin prepared in rabbits). After incubation for two hours at 37° C. (water bath), the tubes were examined for the presence or absence of a precipitate at the interface of the reactants. The usual serum-saline and antigen-saline controls were included with each test.

RESULTS

The results recorded in Table I demonstrate that serologically reactive globulin to a variety of antigens can be recovered by the method described above. Generally the titration end points of the untreated serum and the reconstituted globulin were identical and in no instance was the titer of the reconstituted globulin more than two doubling dilutions lower than the titer of the untreated serum sample.

TABLE I. SEROLOGIC REACTIVITY OF RABBIT AND HUMAN GLOBULINS PRECIPITATED BY 18 PER CENT SODIUM SULFATE

SERUM SOURCE	ANTIBODY DEMONSTRATED	SEROLOGIC TECHNIQUE	TITER*	
			UNTREATED SERUM	RECONSTITUTED GLOBULIN (ORIGINAL SERUM VOLUME)
Rabbit	Sheep cell hemolysin	Comp.-Fix.	4,000	4,000
	Virus-modified human rbc	Tube agglutination	2,048	512
	Brucella agglutinin	Rapid slide	200	200
	Sal. typhosa "O"	Rapid slide	3,200	3,200
	Sal. typhosa "H"	Rapid slide	320	320
	Developing serum	Tube agglutination	128	32
Human	Mumps virus inhibitor	Hem.-Inhibition	512	512
	Blood group B agglutinin	Tube agglutination	32	16
	Sal. typhosa "O"	Rapid slide	320	80
	Sal. typhosa "H"	Rapid slide	160	160
	Syphilitic antibody	V.D.R.L.	10	10
	Psittacosis virus antibody	Comp.-Fix.	320	320

*Titers expressed as reciprocals of serum dilutions.

The choice of 18 per cent sodium sulfate[4] as the final concentration for precipitation was based on preliminary experiments with different salt concentrations. Twenty-six per cent sodium sulfate reportedly precipitates all globulins,[3] but under the conditions of our experiment we were unable to centrifuge out the precipitate formed at that concentration. Nineteen per cent sodium sulfate,[3] which presumably precipitates both beta and gamma globulin, resulted in a satisfactory antibody yield but was somewhat more difficult to centrifuge from suspension than was the 18 per cent fraction. Fifteen per

cent sodium sulfate produced a precipitate which, although readily centrifuged, was not consistently as reactive as the 18 per cent fraction. The 15 per cent fraction is considered to be predominantly gamma globulin[3] and the lessened reactivity observed in the preliminary experiments may have been attributable to the test antibodies being present in other globulin fractions.

To establish that only globulins were precipitated by 18 per cent sodium sulfate, the supernatant fluids and reconstituted globulin fractions from such precipitations were examined serologically and by paper strip electrophoresis.

As can be seen in Tables II and III, both the precipitin reactions and the electrophoretic patterns of the 18 per cent fractions indicate that no albumin was present in the reconstituted globulins (for purposes of comparison 15 per cent fractions were analyzed similarly). Both the serologic and electrophoretic analyses demonstrated that some globulin was present in the supernatant fluids. Moreover, the reconstituted globulins did not consist of a single globulin fraction as determined from the electrophoretic patterns.

TABLE II. EXAMINATION OF FRACTIONATED SERUM BY PRECIPITIN TESTS

	PRECIPITIN RING REACTION				
		SERUM TREATED WITH 15% Na_2SO_4		SERUM TREATED WITH 18% Na_2SO_4	
ANTIBODY	UNTREATED HUMAN SERUM	RECONSTITUTED GLOBULIN	SUPERNATANT FLUID	RECONSTITUTED GLOBULIN	SUPERNATANT FLUID
Antihuman serum (antiglobulin and antialbumin)	+	+	+	+	+
Antihuman albumin	+	-	+	-	+

TABLE III. ELECTROPHORETIC EXAMINATION OF SERUM FRACTIONS

ELECTROPHORETIC FRACTION	15% Na_2SO_4		18% Na_2SO_4		NORMAL SERUM
	GLOBULIN	SUPERNATE	GLOBULIN	SUPERNATE	
Albumin	-	++++	-	++++	++++
α_1 globulin	-	++	-	++	++++
α_2 globulin	-	++	-	++	++++
β globulin	++	+	+++	++	++++
γ globulin	++++	trace	++++	trace	++++

DISCUSSION

Salting-out procedures for the recovery of antibody-globulins are relatively simple techniques, but as a rule they are time consuming due to the one or more steps involving dialysis. The present report suggests that dialysis, to remove residual salt from the precipitated globulins, may be avoided by "washing" the surface of the precipitate with distilled water. The sodium sulfate remaining after this step is not present in sufficient quantity to interfere with serologic demonstration and the amount of globulin lost by the "washing" is too minute to affect the antibody titer as was demonstrated with a variety of antigen-antibody systems.

The excellent recovery of antibody-globulin from small quantities of sera (1.0 ml.) permits this method to be used in laboratories where large numbers

of serum samples (either human or rabbit) are routinely examined, and where it is of practical and theoretical importance to test many samples simultaneously.

Although it has been the practice in these laboratories to reconstitute the globulin to the original serum volume, this is not at all necessary. Concentration of globulin may be readily accomplished by limiting the quantity of distilled water employed for the globulin reconstitution, thereby enhancing the opportunity for detecting low levels of antibody present in the untreated serum.

In other serologic studies (to be reported later) the fractionation technique also has proved valuable as a means of removing albumin from test serums in which it was observed to be an interfering factor.

SUMMARY

A method has been described for rapidly recovering serologically-reactive globulin, free of albumin from serum samples of 1.0 ml. or less. The method depends on the precipitation of the globulin with sodium sulfate. Dialysis is avoided by carefully washing the precipitated and centrifuged globulin with double distilled water before reconstitution. Tests with antisera to a variety of antigens show the procedure to have wide application.

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METHOD FOR THE QUANTITATIVE ESTIMATION OF 1-CYCLOHEXYLAMINO-2-PROPYLBENZOATE (CYCLAINE) AND DERIVED PRODUCTS IN BIOLOGIC TISSUES

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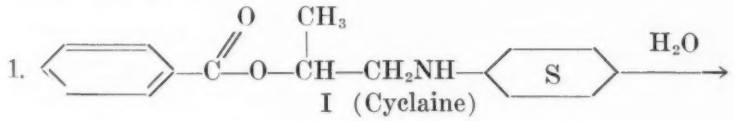
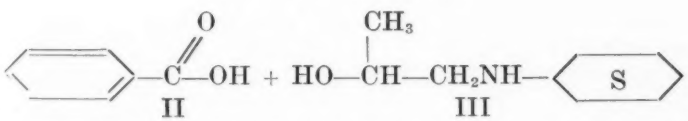
SUBSEQUENT to extensive pharmacologic and toxicologic studies, Cyclaine*[1-4] has been found to be a useful and versatile local anesthetic agent. Considerable clinical literature on its use now exists, although analytical procedures for its estimation in body fluids have not been published previously. The long duration of action and the effectiveness of Cyclaine at low concentrations enhance the need for such a method in clinical studies of the properties of this agent.

The following analytical procedures have been developed by the modification of those described by Brodie and associates[5, 6] and have been employed successfully in our laboratory studies.

We have found that Cyclaine is very slowly hydrolyzed by rat liver extracts to yield benzoic acid and the amino alcohol (III, equation 1). In addition, the rearrangement to an amide (IV, equation 2) occurs in alkaline solutions[7] and in restored human plasma. In plasma, this rearrangement has shown the following characteristics: (a) 50 to 75 per cent of Cyclaine is converted to the amide and (b) the rearrangement occurs very rapidly and is probably nonenzymatic. In view of the foregoing observations, any study of the fate of Cyclaine, in vivo or during exposure to tissues or tissue extracts in vitro, must account not only for the ester and its hydrolytic products but also for the amide. The following procedure is designed to do this. The development of the method was aided greatly by virtue of having compounds I, III, and IV supplied in pure form as reference agents.†

1. 

I (Cyclaine)



II III

From the Merck Institute for Therapeutic Research, West Point, Pa.
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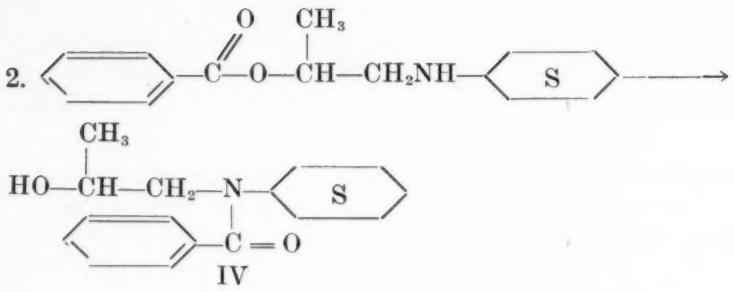
*Cyclaine is the trademark of Merck & Co., Inc., for its brand of 1-cyclohexylamino-2-propylbenzoate, the nonpropriety name of which is hexylcaine.

†These compounds were supplied to us through the courtesy of Dr. James M. Sprague, Director of the Department of Medicinal Chemistry.



IV

PRINCIPLE

The colorimetric quantitation procedure[5, 6] utilized in this method works well with the amino alcohol (III) liberated by hydrolysis of either Cyclaine (I) or the rearrangement product (IV). For convenience, the latter is referred to as "Cyclaine amide," or simply as the amide derived from Cyclaine. Neither Cyclaine nor the amide will yield a color in the aforementioned colorimetric method. The amide is much more soluble in ethylene dichloride than in water. These observations were utilized for the development of a method to determine quantitatively each of the three compounds (I, III, and IV) when present as a mixture.

If an aliquot of plasma, urine or liver extract containing Cyclaine, the amino alcohol and the amide is alkalinized and extracted with ethylene dichloride, all three of these compounds are extracted into the organic solvent. If a portion of the ethylene dichloride is removed and shaken with dilute HCl, the Cyclaine and the amino alcohol are extracted into the acidic aqueous phase while the amide remains in the organic phase. The aqueous phase is alkalinized and divided into two portions. One portion is analyzed for free amino alcohol. The other portion is heated to hydrolyze the Cyclaine. This yields an increase in amino alcohol content equivalent to the Cyclaine. This increase is determined by colorimetric analysis of the hydrolysate. To determine the amide, an aliquot of the ethylene dichloride extract is mixed with alkali and heated. After the organic solvent is distilled off, the heating is continued in order to hydrolyze the amide. The hydrolysate contains amino alcohol in amounts equivalent to the amide content before hydrolysis.

The general procedure for the quantitative determination of the amino alcohol is as follows: By shaking an alkaline solution of the compound with ethylene dichloride the basic alcohol is extracted into the organic phase. An aliquot of the ethylene dichloride is shaken with a saturated solution of methyl orange and boric acid. Through salt formation with the base, methyl orange will dissolve in the organic phase in amounts equivalent to the contained base. The excess methyl orange is completely removed by aspiration. An aliquot of the ethylene dichloride is transferred to a colorimeter tube, and the color of the dye is developed by the addition of an alcoholic sulfuric acid solution. The optical density of the sample at 540 mμ is determined by reading it in an Evelyn colorimeter, or another suitable instrument.

Reagents.—

1. 0.2 N Hydrochloric acid.
2. 1.0 N, 1.2 N and 2.0 N Sodium hydroxide.
3. Saturated solution of the sodium salt of methyl orange.*
4. 0.5 M Boric acid.*
5. Alcoholic sulfuric acid solution: 2 per cent by volume of concentrated sulfuric acid in absolute ethanol.

*The saturated methyl orange is washed several times by shaking with ethylene dichloride. The methyl orange reagent is prepared just before use by dilution of this solution with an equal volume of the boric acid solution. This yields a supersaturated solution of methyl orange, which is stable for approximately one hour.[6]

6. Ethylene dichloride: technical grade purified by successive washings with 1 N sodium hydroxide, 1 N hydrochloric acid, and three washings with distilled water. 1.5 per cent by volume of isoamyl alcohol (similarly purified) may be added to minimize adsorption of compounds onto the glass* surfaces.[6]

PROCEDURE

I. Preparation of Sample.—Add 2 ml. of plasma, urine, or tissue extract and 2 ml. of 2 N sodium hydroxide to 6 ml. of ethylene dichloride in a shaker bottle. Stopper and shake vigorously for 15 minutes. Transfer to a graduated centrifuge tube and centrifuge for 10 minutes. Remove the aqueous phase by aspiration. Transfer 4 ml. of the ethylene dichloride into shaker bottles containing 4 ml. of 0.2 N hydrochloric acid. Shake vigorously for 15 minutes. Transfer to a graduated centrifuge tube and centrifuge for 10 minutes. Aliquots of each phase are collected separately for further treatment as follows:

1. Acidic aqueous phase: Collect 3 ml. and add it to 3 ml. of 1.2 N sodium hydroxide. Mix well. Take a 2 ml. aliquot for determination of amino alcohol. Transfer 3 ml. of the remaining solution to a test tube with a 3 ml. graduation mark. Place a small funnel in the top of the tube and heat in a boiling water bath for 60 minutes. Cool, remove the funnel, and make the volume back up to 3 ml. with water, if necessary. Take a 2 ml. aliquot for determination of the amino alcohol.

2. Ethylene dichloride phase: Transfer 3 ml. of the ethylene dichloride phase to a test tube with a 3 ml. graduation mark. Add 3 ml. of 1 N sodium hydroxide. Place the tube in a boiling water bath. When all of the organic solvent has distilled off, place a small funnel in the neck of the tube to minimize loss of the aqueous volume, and continue heating for 60 minutes. Cool, and remove the funnel. If necessary, add water to make the volume 3 ml. Take a 2 ml. aliquot for determination of its amino alcohol content.

II. Determination of Amino Alcohol.—Transfer 2 ml. of alkaline sample to shaker bottle containing 20 ml. of ethylene dichloride. Stopper and shake vigorously for 15 minutes. Transfer the contents of the shaker bottle to an 8 inch test tube and centrifuge for 20 to 30 minutes. Completely remove the aqueous phase by aspiration. Transfer 15 ml. of the ethylene dichloride to a shaker bottle containing 0.3 ml. of freshly prepared methyl orange reagent. Shake vigorously for 25 minutes. Transfer the mixture to a test tube and centrifuge for 20 to 30 minutes. *Completely* remove the excess methyl orange by aspiration. Transfer 10 ml. of the lightly-colored ethylene dichloride into colorimeter tubes containing 2 ml. of alcoholic sulfuric acid. After 10 minutes, the tubes are read in an Evelyn colorimeter at 540 mμ. A solution of 10 ml. of ethylene dichloride and 2 ml. of alcoholic sulfuric acid is employed to adjust the colorimeter setting at 100 per cent transmission. "Tissue blank" samples can be prepared from tissue samples collected before drug administration; these should not read less than 95 per cent transmission.

Standard curve: A reference solution of amino alcohol can be prepared from the amino alcohol itself or by alkaline hydrolysis of a standard solution of Cyclaine. If a standard solution of the latter is prepared, aliquots can be hydrolyzed by adding an equal volume of 1.2 N sodium hydroxide and heating in a boiling water bath for one hour. Samples of the hydrolysate can be taken directly into the procedure, as described, for the determination of amino alcohol. The recoveries are essentially quantitative. The samples should range from 8 to 30 γ of amino alcohol per milliliter. In this range Beer's Law is followed and a constant (K) can be calculated, i.e., $K = \frac{C}{L}$, where C = concentration (in γ/ml.) and L = 2 − log G. G is the galvanometer reading. We obtained a K value of 58.7. In repeated determinations this should not vary more than ± 0.5.

*All glassware should be washed thoroughly with hot distilled water, since some detergents will cling to the glassware and can cause erratic results.

When mixed with fresh rat liver extracts,* dog urine, dog plasma, or restored human plasma,† Cyclaine or the amino alcohol could be determined with recoveries of 94 to 102 per cent. The recoveries of the amide were somewhat lower, ranging from 90 to 97 per cent. When Cyclaine is added to plasma it is necessary to determine the ester, the amide, and the amino alcohol in order to calculate a recovery. Amino alcohol concentrations of approximately 1 γ/milliliter can be measured, but the accuracy is not as great as can be obtained with levels such as we have used for determination of a standard curve.

RESULTS

The hydrolysis of procaine and Cyclaine by liver extracts is shown in Table I. In the system we employed it is obvious that procaine is cleaved much more rapidly than is Cyclaine.

Table II summarizes some data obtained following the administration of procaine and Cyclaine to dogs. The 6-hour urine recoveries of 5 to 6 per cent of the administered dose of procaine is about the recovery one might anticipate from the work of Terp[8] and Brodie and associates.[6] Although the experiments with procaine were terminated at 6 hours, the plasma levels and the urinary excretion of the ester have already decreased in a pronounced fashion at the 4- to 6-hour period. The maintenance of appreciable plasma levels of p-aminobenzoic acid (PAB) and the resultant prolongation of its urinary excretion can be attributed, in large part, to the low renal clearance of this acid.[9] The 6-hour urinary excretion of procaine per se and PAB accounts for over half of the administered drug. It seems certain that substantially higher recoveries would have been obtained if 24-hour urine collections had been made.[6]

TABLE I. RATES OF HYDROLYSIS OF PROCAINE AND CYCLAINE BY RAT LIVER EXTRACTS

COMPOUND	TIME (MIN.)	PER CENT HYDROLYZED	
		EXPERIMENT 1	EXPERIMENT 2
Procaine	0-1	0.2	—
	5	6.3	36.5
	15	35.1	50.2
	30	56.8	60.4
	60	71.9	76.4
	120	90.9	84.9
Cyclaine	0-1	—	—
	15		2.6
	30	11.3	2.6
	60	11.3	2.6
	120	39.0	8.6
	240		16.1
	360		20.3

Twenty-eight milliliters of rat liver extract was incubated with 2 ml. of procaine or Cyclaine. The latter contained 0.5 mg. of local anesthetic per milliliter. The temperature was 26° C. The rates of hydrolysis were determined by the rate of liberation of the amino alcohols.

When Cyclaine was given intravenously at 5 mg. per kilogram, there was little or no demonstrable ester in the plasma. However, there was a very uniform persistence of significant amounts of amino alcohol in the plasma over the whole of a 24-hour period. This occurred despite the continued urinary

*Rat liver extracts were tested for their ability to cleave a number of local anesthetics, including Cyclaine. The extracts were prepared by blendorizing fresh rat liver with 4 volumes of 0.15 M sodium phosphate buffer, pH 7.4, in a Waring blendor. After centrifuging for 20 minutes at 3,000 g, the supernatant was collected for use.

†Lyophilized human plasma restored by the addition of distilled water.

TABLE II. PLASMA LEVELS AND URINARY EXCRETION OF PROCAINE, CYCLAINE AND DERIVED PRODUCTS FOLLOWING ADMINISTRATION TO DOGS

COMPOUND	DOSAGE MG./KG.	TOTAL DOSE (MG.)	TIME (HR.)	PLASMA LEVELS (MG./100 ML.)			URINARY EXCRETION (MG./PERIOD)		
				Procaine	*PAB*		*Procaine*	*PAB*	
Procaine	20, IV	266	1/6	0.61	0.73		—	—	
			½	0.41	0.68		1.63	2.28	
			1	0.20	0.78		5.28	10.03	
			2	0.10	1.34		5.08	20.80	
			4	—	0.23		3.38	31.00	
			6	—	0.41		0.74	12.96	
				Total mg.			16.11	77.07	
				Per cent of total dose			6.06	49.8	
Procaine	20, IV	386	1/6	0.63	0.38		1.34	0.78	
			½	0.27	0.21		3.07	4.14	
			1	0.15	hemolysis		4.14	10.60	
			2	0.05	hemolysis		7.09	25.05	
			4	0.03	0.02		3.98	44.40	
			6	0.02	0.01		1.02	20.04	
				Total mg.			20.64	105.01	
				Per cent of total dose			5.35	46.9	
				Cyclaine	*Cyclaine alcohol*	*Cyclaine amide*	*Cyclaine*	*Cyclaine alcohol*	*Cyclaine amide*
Cyclaine	5, IV	72	1/6	—	0.110	0.065	—	—	—
			½	—	0.130	—	—	1.975	0.912
			1	0.069	0.110	—	—	1.282	0.229
			2½	—	0.087	—	—	1.490	0.070
			4	—	0.110	0.087	1.75	0.920	0.445
			6	—	0.087	—	—	0.713	0.133
			6 to 24	—	0.110	—	—	7.946	1.557
				Total mg.			1.75	14.326	3.336
				Per cent of total dose			2.4	30.7	4.6
Cyclaine	5, IV	70	1/6	—	0.084	—	—	—	—
			½	—	0.084	0.120	0.206	1.340	0.268
			1	—	0.057	—	—	0.600	0.030
			2	—	0.057	0.055	—	0.270	—
			4	—	0.057	—	—	0.530	0.132
			6	—	0.028	0.120	—	0.440	—
			6 to 24	—	0.057	0.033	—	7.124	0.548
				Total mg.			0.206	10.304	0.978
				Per cent of total dose			0.29	22.0	1.4
Cyclaine	50, PO	980	½	—	0.127	—	—	0.273	0.014
			1	—	0.365	—	—	7.104	0.063
			2	0.873	0.320	—	0.850	25.116	0.449
			4	—	0.429	—	4.370	41.180	0.040
			6	0.216	0.062	—	—	19.600	—
			6 to 24	0.023	0.042	—	—	26.900	—
				Total mg.			5.220	120.173	0.566
				Per cent of total dose			0.53	17.6	0.57
Cyclaine	75, PO	1,240	½	—	0.534	—	1.90	5.18	—
			1	—	0.503	—	9.65	10.92	—
			2	—	0.838	—	42.80	27.03	—
			4	0.040	0.275	—	36.60	50.09	—
			6	—	0.305	—	6.82	18.23	—
			6 to 24	—	—	—	—	14.45	—
				Total mg.			97.77	125.90	0.0
				Per cent of total dose			7.9	15.6	0.0

excretion of considerable amounts of this Cyclaine derivative. The renal excretion of amino alcohol during the 6- to 24-hour period is roughly equivalent to its excretion during the first 6 hours after drug administration. The amide

derived from Cyclaine could be detected in both plasma and urine. The latter compound, however, constituted only a minor portion of the recovery of derivatives of the administered drug. In 24 hours, substantially less than half of the dose of Cyclaine could be accounted for as Cyclaine or derivatives in urine. More complete data might have been obtained had we raised the dose to 7 mg. per kilogram; the calculated convulsive dose for 50 per cent of the dogs (CD_{50}), as determined by Beyer and associates,[4] was 10 mg. per kilogram. When given orally, Cyclaine could be detected in the plasma and significant amounts of the ester were found in the urine, especially at the higher dose. This would indicate not only a reasonable degree of absorption into systemic circulation but also a limited rate of hydrolysis. The findings with respect to the amino alcohol derivative are similar to the data obtained after intravenous administration of the drug.

The foregoing data are in harmony with studies[1-4] demonstrating a moderately prolonged pharmacologic effectiveness at low concentrations.

SUMMARY

A method has been described for the quantitative determination of Cyclaine and several derivatives thereof in plasma, urine, and liver extracts. Comparative data on Cyclaine and procaine, in vitro and in vivo, have been presented.

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THE AGAR DIFFUSION PRECIPITIN TEST AS A MEANS OF RECORDING EVIDENCE OF MEAT ADULTERATION

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ALTHOUGH the classical tube precipitin test for detecting adulteration of beef intended for human consumption is simple, rapid, and specific, it does not provide an exhibit which is easily presented at a medicolegal proceeding. It appeared, therefore, that if a specific reaction could be demonstrated by the agar diffusion technique (Ouchterlony),[1, 2] a photographic record of the laboratory determination readily could be made.

MATERIALS AND METHODS

Preparation of Plates.—One per cent agar (Difco) was dissolved in distilled water, and Merthiolate 1:10,000 was added. Clarification of the agar was not necessary. The melted agar was poured into new, clean, unscratched 100 mm. Pyrex Petri dishes using 25 ml. per plate. Filter paper was inserted in the top of each plate to prevent condensation on the glass. After the agar had solidified, reservoirs were made in the desired positions by placing the plate over a guide drawn on paper and cutting out disks of agar with a No. 6 cork borer.[3] The disks were removed with the aid of a wood applicator shaped into a small spatula. Such reservoirs were 13 mm. in diameter and had a capacity of 0.2 ml. The distance between reservoirs destined to contain immune serum and antigen, respectively, was 7 mm., thereby providing sufficient space for a reaction to be observed without unduly prolonging the time of appearance of the reaction.

Preparation of Meat Extracts.—Ten grams of ground meat was extracted with 10 ml. of saline in the refrigerator overnight. Sufficient clarification of the extract was obtained by filtration through paper or by centrifugation. Merthiolate, 1:10,000, was added to the extract to prevent bacterial growth during the observation of the plates.

Conducting the Test.—Two-tenths milliliter of undiluted immune serum was pipetted into the center reservoir. The same amount of extract or dilution of extract was placed in the peripheral reservoirs. Care was taken to avoid letting the fluid overflow on the agar surface since this interferes with photographic reproduction of the reaction. After the filling of the reservoirs, the plates were placed in a desiccator, or other closed container, in a level position and examined daily for lines of precipitation.

Recording the Reaction.—Direct-contact photographic prints of the reactions were made by placing the plates on high contrast paper (Kodak Medalist, F-4, was used in making the prints reproduced here). With the covers removed the plates were exposed to a 6 watt bulb at a distance of 53 inches for 1 second. The prints then were developed in the usual way.

RESULTS

In order that the sensitivity of the test might be determined, plates were prepared in which rabbit antihorse serum was tested against dilutions of horse

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Fig. 1.—*A*, Reaction between rabbit antihorse serum and diminishing concentrations of horse meat extract after 24 hours. RAHS indicates rabbit antihorse serum; numbers indicate per cent concentrations of horse meat extract. *B*, Same as *A* after 48 hours.

Fig. 1.—*C*, Reaction between antihorse serum and extracts of mixtures of horse meat and ground beef after 24 hours. Numerators indicate per cent of horse meat in each mixture; denominators indicate per cent of ground beef. *D*, Same as *C* after 48 hours.

meat extract (Fig. 1, *A* and *B*). After 24 hours, lines of precipitation were readily visible with undiluted extract and with dilutions containing 25 and 6.25 per cent extract. No reaction was visible with 1.5 per cent extract even after 48 hours at which time the bands present in 24 hours were intensified and a second band was visible with the undiluted extract. The sensitivity of this reaction compared favorably with the conventional tube precipitin test in which the lowest concentration of extract showing a ring was 6.25 per cent, although 1.5 and 0.37 per cent showed traces of precipitate on standing overnight. When the undiluted rabbit antihorse serum used was tested against horse serum in a tube test, the highest dilution of horse serum showing a ring was 1:16,000, while a 1:32,000 dilution showed a precipitate on standing overnight.

To further explore the usefulness of the procedure, mixtures of ground horse meat and beef were prepared, extracted, and tested against rabbit antihorse serum (Fig. 1, *C* and *D*). Dense lines of precipitation were obtained with extracts of pure horse meat and of a mixture containing 25 per cent horse meat. A faint but definite line occurred with an extract of beef adulterated with 5 per cent horse meat. No trace of reaction appeared with an extract of pure beef. The halos appearing around the reservoirs containing beef extract are due to the presence of fat, which, if excessive, might interfere with the detection of faint lines.

DISCUSSION

It is not usually difficult to demonstrate meat adulteration by means of the conventional precipitin test, provided appropriate species specific immune sera are available. The agar diffusion precipitin test compares favorably with the tube test in sensitivity and appears to be sufficiently sensitive to detect any economically worth-while degree of adulteration. The agar diffusion reaction is not as rapid since it requires about 24 hours for a marked reaction to develop. This delay, however, would probably not be critical in most instances. The agar diffusion test has a distinct advantage in that the result can be recorded easily by means of a direct print on photographic paper to provide a permanent record and a record which can be exhibited as evidence at any time after the actual test is performed.

CONCLUSIONS

1. The agar diffusion precipitin test is a practical means of detecting meat adulteration.

2. The method has the advantage of yielding a permanent photographic record of the reaction.

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